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13. ABSTRACT

Exposure to HD of a selected number of phylogenetically different cell types (human skin keratinocytes, J774 cells, CHO-K1 cells, thymocytes and chick neurons) resulted in concentration-dependent cytotoxicity. HD induced apoptosis was shown to be common to all cell types tested as illustrated by annexin staining, production of DNA fragments, soluble DNA assay, morphology (acridine/ethidium bromide staining) and positive TUNEL reaction. Apoptosis was prevalent at lower concentrations, while necrosis was relatively more evident at higher HD concentrations. Calcium levels were elevated in HD exposed human keratinocytes, J774 cells and CHO-K1 cells at concentrations of HD that ultimately caused cell death. HD induced cytotoxicity (apoptosis) was unaffected by manipulation of calcium levels prior to exposure, suggesting no cause-effect relationship in keratinocytes. The role of calcium in other cell types is currently being examined. Studies using molecular and immunohistochemical techniques to identify ATP receptor types in HD sensitive cells revealed novel findings, including the presence of P_2X_1 and P_2X_7 receptors on vas deferens, and on immature thymocytes. ATP induced calcium influx in synaptosomes was demonstrated following activation of a novel CNS P_2X_7 receptor. HD did not potentiate this influx. The effects of caspase inhibitors and the role of cytoprotective genes in HD toxicity are currently being investigated.

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TABLE OF CONTENTS

INTRODUCTION

This cooperative agreement proposed to elucidate a possible relationship between the potentiation of P_2X ionotropic membrane ATP receptors by sulphur mustard and the cytotoxicity of these compounds. This report summarizes the first year's results.

BODY OF REPORT

1. BACKGROUND

The hypothesis that originally formed the basis for the work proposed in this cooperative research project, was based on the observation that sulphur mustard (HD) potently and irreversibly potentiated the effect of ATP on smooth muscle preparations (Lundy *et al.,* 1998) and also caused a sustained elevation of free intracellular Ca^{2+} ($[Ca^{2+}]_{INT}$) (Hamilton *et al.,* 1999). These results were intriguing for three reasons: 1: It appeared that both ATP and HD interacted with P_2 receptors; 2: Both extracellular ATP and HD can kill cells; and 3: Both ATP and HD cause an increase in $[Ca^{2+}]_{INT}$.

Recent evidence suggests that extracellular ATP is a broad spectrum cytotoxic agent that kills cells and promotes apoptosis by activation of distinct cell surface P_2 purinoceptors (Burnstock, 1998). Some of these receptors, notably the P_2X sub-family are ionotropic channels, acting, for example, to transport calcium through the cell membrane causing a variety of physiological effector responses, such as muscle contraction or nerve transmission (Burnstock, 1998; Evans *et al.,* 1992). At least two of the ATP receptors in this sub-family, in addition to their physiological roles, are also thought to mediate programmed cell death. The P_2X_1 subtype has been proposed to initiate apoptosis following activation and has been shown to be upregulated in cells undergoing this phenomenon (Chvatchko *et al.,* 1996; Zambon *et al.,* 1994). This particular receptor has marked sequence homology (Valera *et al.,* 1994; Brake *et al.,* 1994) to the product of the gene known as RP-2, which has been shown to encode for apoptosis (Owens *et al.,* 1991). The P_2X_7 receptor, on the other hand, is not only an ionotropic receptor which is permeable to ions such as calcium, but is also coupled to a non-specific membrane pore, which, following activation by ATP and its analogues, opens to allow the entrance into the cytoplasm of large molecules, up to about 800 daltons (Wiley *et al.,* 1996). The literature suggests that activation of this receptor may initiate cell lysis (necrosis), or what has also been termed colloidal osmotic lysis. However, this receptor has not, to our knowledge, been specifically linked to apoptosis. It is believed that the cytotoxic activity of ATP is generally the result of the stimulation of one or both of these receptors and that the specific type of cell death; either apoptosis or necrosis, is dependent on the type of receptor present on the target cell (Papirmeister *et al.,* 1991).

Similar to ATP, HD is also a broad spectrum cytotoxic agent, albeit much more potent and whose mechanism of cytotoxic activity is not clear (Papirmeister *et al.,* 1991). We proposed to examine the mechanism of the cytotoxic activity of HD and its relationship to P_2 receptors. Because our original observation found that these two cytotoxic agents markedly potentiate one another on a physiological response in smooth muscle preparations known to be mediated by the P_2X_1 receptor, and because both P_2X_1 and P_2X_7 ATP receptors mediate cell death, we hypothesized that the P_2X_1 receptor, and possibly the P_2X_7 receptor, might mediate some of the cytotoxic effects of HD (Lundy *et al.,* 1998, Hamilton *et al.,* 1999).

It is well documented that the P_2X_1 extracellular receptor which mediates the contraction of the vas deferens and the bladder, the two tissues on which ATP/HD potentiation was first observed, functions as an ionotropic channel when activated, allowing the influx of calcium into the interior of the cell (Valera *et al.,* 1994). Furthermore, ATP activated calcium influx is widespread in a number of tissues in which ATP also causes cell death (Dowd, 1995; McConkey, *et al.,* 1989). The implication is that ATP induced cell death may be triggered by calcium influx or a rise in $[Ca^{2+}]_{INT}$, although good evidence of a cause-effect relationship is lacking (Papirmeister *et al.,* 1991). There is now also considerable speculation that calcium may play an important role in the initiation of HD induced cell death (Papirmeister *et al.,* 1991; Hua *et al.,* 1993; Hamilton *et al.,* 1999). The role of calcium in the initiation of cell death through calcium mediated enzyme activation is very well documented (Orrenius and Nicotera 1987) and suggested to us that calcium overload, as a result of stimulating ATP receptors either by ATP, by ATP and HD together or indeed by HD itself, could activate calcium dependent proteases, phospholipases and endonucleases, and thereby initiate apoptosis, necrosis or perhaps both types of cell death.

This yearly report documents the steps we have taken in the first year of the study to determine the mechanism of the cytotoxic activity of HD and ATP and reports our findings with regard to:

(a) the unequivocal demonstration of the induction of apoptosis/necrosis in a number of cell types (chick neurons, CHO-K1, J774, human keratinocytes) exposed to HD at concentrations which induce cytotoxicity. We examined the molecular basis of the toxicity of HD and ATP using a variety of techniques that differentiate apoptosis from necrosis.

(b) the determination of the P_2 receptor subtypes resident on those cells listed in the previous paragraph.

(c) the testing of the hypothesis that HD and ATP do indeed lead to increased intracellular free Ca^{2+} levels. Our goal is to provide critical evidence for or against a cause-effect link between the toxicity of HD and HD induced calcium levels in the cell types listed above. Initial studies are reported in which HD induced elevation of calcium is demonstrated, as well as its relationship to the resultant HD toxicity. Finally, preliminary studies are reported that identify types of membrane P_2 receptor types (see b above) and to determine whether these receptors function as ion channels.

2. METHODS

2.1 Cell Culture and Cytotoxicity Assays

2.1.1 Human Skin Keratinocyte Culture

Primary cultures of human skin keratinocytes were prepared from neonatal foreskins. Tissue was obtained on the day of circumcision and incubated at $4^{\circ}C$ for 24 h in 25 U/ml dispase (Collaborative Research, Bedford, MA). The epidermis was removed and the cells segregated by a further 5 min incubation in 0.25% trypsin at $37^{\circ}C$. The trypsin was deactivated by the addition

of 20% serum in medium and the cell suspension was centrifuged. The cell pellet was resuspended in Keratinocyte Serum Free Medium (KSFM, Gibco BRL, Grand Island, NY) and filtered through 70 μm nylon mesh. 75 cm^2 flasks were seeded at 5 x 10^5 cells/8 ml KSFM supplemented with gentamicin (50 μg/ml) and Fungizone (0.25 μg/ml) and incubated in a 37°C humidified incubator in a 5% CO$_2$/95% air atmosphere. Cultures were re-fed every 2 - 4 days, as required. First passage cultures were seeded from log growth primary cultures at a density of 1000 cells/well in 96-well Costar multiwell plates or in 35 mm culture dishes at 30,000 cells/dish.

2.1.2 Chick Embryo Neuron Culture

Cultures of chick embryo neurons were prepared as previously described (Weiss and Sawyer, 1993) and routinely plated at a density of 100,000 cells/well in 96 well titer plates coated with 0.1 ml/well of 12.5 μg/ml polylysine in water. The cells were seeded in 0.1 ml of 5% horse serum in mMEM supplemented with streptomycin (100 μg/ml), penicillin (100 IU/ml) and Fungizone (2.5 μg/ml).

2.1.3 Thymocyte Culture

Three to four week old Sprague-Dawley rats were killed by CO$_2$ inhalation and the thymuses were removed aseptically. The tissue was rinsed twice with sterile PBS and then cut into smaller pieces with scissors. The tissue was rinsed twice more and then placed into room temperature trypsin (0.25%, 5 ml/thymus) just prior to being finely minced with scalpel blades. The suspension was tricturated 20 times and then gently agitated at room temperature for 10 min. The trypsin was deactivated with 20% FCS in DMEM and then centrufuged for 10 min at 1000g. The pellet was resuspended in 10% FCS in DMEM supplemented with streptomycin (100 μg/ml), penicillin (100 IU/ml) and Fungizone (2.5 μg/ml), filtered through 70 μM nylon mesh and counted. Cells were seeded at 1.5 x 10^6 cells/well in 96 well titerplates.

2.1.4 CHO-K1 and J774 Cell Line Culture

Seed cultures of both cell lines were obtained from the American Type Culture Collection. The cells were grown in 10% FCS in DMEM supplemented with streptomycin (100 μg/ml) and penicillin (100 IU/ml) and the medium was changed twice a week. Subconfluent cultures were passaged (1:3) into 75 cm^2 culture flasks or into 96 well titerplates at a density of 2000 (CHO-K1) or 4000 (J774) cells/well.

2.1.5 Chemical Treatment and Cytotoxicity Studies

On the day of chemical treatment the cultures were treated with freshly prepared treatment medium so that the desired final HD concentration was reached at 0.25% ethanol (v/v). The viability of HD-exposed cultures was determined at 24 or 48 h (keratinocytes). In experiments which assessed the effects of BAPTA-AM, ionomycin or thapsigargin on HD

toxicity in human keratinoyctes, the compounds were dissolved in ethanol or DMSO (BAPTA-AM) and administered to the cultures 1 h prior to HD treatment. In studies where external calcium was also varied, 1 h before drug treatment, the cultures were aspirated, rinsed twice with 200 µl of phosphate buffered saline and then re-fed with calcium-free medium that had been adjusted to the desired calcium concentration with calcium chloride. In these experiments Fura-2 was not used to quantitate the external calcium and therefore these values represent the *nominal* calcium concentrations. In all experiments, test drugs were left in the cultures for the full 48 h test period. The test drug vehicles (ionomycin; 0.046% ethanol, thapsigargin; 0.1% ethanol, BAPTA-AM; 0.15% DMSO) had no effect on the viability of the cultures, even in combination with the ethanol used as the HD vehicle. To assess cytotoxicity, alamarBlue (AccuMed International Inc., Westlake, OH) was added (10%, v/v) and the cultures were allowed to incubate with the dye for the last 2 - 5 h of the treatment time period. This assay is based on the reduction of a dye by viable cells to a coloured species which can be measured by absorbence or fluorescence, and has also been found to yield similar results compared to a number of more commonly used dyes and indicators (Fields and Lancaster, 1993). The absorbences (570 nm - 600 nm) were then read on a Thermomax titerplate reader (Molecular Devices, Sunnyvale, CA). Median lethal concentration (LC_{50}) values were determined graphically from experiments utilizing 6 wells per data point. All values represent data obtained from at least 3 separate experiments. Sulphur mustard was prepared by the Hazard Avoidance Section, Defence Research Establishment Suffield at greater than 99% purity.

2.2 Calcium Measurements

Studies to assess the effect of HD on FURA fluorescence were performed using the cell impermeant pentapotassium salt of Fura-2 (0.4 µM) in high (1 mM, pH 7.4) and relatively low (225 nM, pH 7.4) Ca^{2+} buffer solutions (Molecular Probes, OR). Fluorescence of Fura-2 was monitored with a Delta-Scan 1 dual excitation/emission fluorometer with front-surface optics sample compartments (model D105; Photon Technology International Inc., Princeton, NJ). The output from the xenon lamp was directed to two excitation monchromators with wavelengths set at 340 and 380 nm (5 nm bandpass), respectively, using a chopper wheel. Output from the excitation monochromators was focused on a 1 cm^2 quartz cuvette by a fibre-optic cable and the fluorescence collected through an emission monochromator at 510 nm (5 nm bandpass) using a photomultiplier tube. Experiments were performed after loading the cells with the membrane permeant form (Fura-2 AM) of the calcium sensitive dye Fura-2 (Grynkiewicz *et al.*, 1985; McDonough and Button, 1989). The cell suspension was rinsed three times with HEPES buffered Hank's balanced salt solution (pH 7.4, 1 mM nominal Ca^{2+} concentration). The cells were then incubated in the dark with 3 µM Fura-2 AM in DMSO/pluronic-127 for 45 min at room temperature. After incubation, the buffer was changed to buffer containing no Fura-2 AM and allowed to equilibrate for least 30 min prior to recording fluorescence signals. The cell suspension (2.4 ml) was placed in a quartz cuvette and measurements were made with continuous stirring in a temperature-controlled cuvette holder at 37°C. HD was added to the cuvettes and the signal re-acquired after a delay of 1 min to eliminate an artefact due to the dissolution of the HD in the buffer. Signals were background subtracted using Fura-2 free cells

for autofluorescence and, where appropriate, solvent addition.

2.3 ATP Receptor Subtypes in Nerve Endings

2.3.1 Preparation of Synaptosomes

Rat cortical synaptosomes were prepared by homogenization of brain cortex in 0.32 M sucrose using six strokes of a teflon/glass homogenizer. The homogenate was the centrifuged at 1000g for 10 min at 4°C. The supernatant was decanted and centrifuged at 2,400g for 25 min, and the resulting pellet (P_2) was resuspended in a HEPES buffered physiological salt solution of the following composition (mM): $C_5H_{14}NO.Cl$, 132; KCl, 5; $MgCl_2$, 1.3; $CaCl_2$, 1.5; NaH_2PO_4 1.2; D-glucose, 10; HEPES, 20; brought to pH 7.4 with Tris base. The protein concentration was measured using the method of Bradford (1976) and adjusted to 1.0-1.5 mg/ml.

2.3.2 Ca^{2+} Influx

Ca^{2+} influx was carried out according to the method of Blaustein (1975) with modifications (Lundy *et al.*, 1991). Synaptosomes were incubated at 30°C in the presence or absence of antagonist drugs for 15 min, at which time a 100 µl aliquot of the synaptosomal suspension was quickly injected into an equal volume of resting buffer (5 mM K^+), depolarizing buffer (25 mM K^+, final concentration), or resting buffer to which ATP or it analogs had been added (final concentration 1 mM), and all containing 0.5 µCi $^{45}Ca^{2+}$ (New England Nuclear, Boston, MA). Basal and K^+ stimulated Ca^{2+} influx was allowed to proceed for 3 sec, and basal and nucleotide-stimulated for 10 to 90 sec, at which time it was stopped by rapid dilution of the buffer with 4 ml ice-cold Ca^{2+}-free buffer containing 4 mM EGTA. Each suspension was rapidly filtered under vacuum through 0.45 µm membrane filters (Gelman Science) using a filtration apparatus (Hoeffer Scientific, San Francisco, CA). The membrane filters were washed twice with 5 ml resting buffer containing excess Ca^{2+}. Membrane filters were allowed to dry, placed in scintillation cocktail and counted on a Wallac 1500 scintillation counter. Assays were carried out in triplicate. Basal influx was subtracted from K^+ or nucleotide-stimulated influx and results expressed as Ca^{2+} influx (nMole per mg tissue). Experimental protocols were designed so that effects of ATP, and five nucleotide analogs could be examined on each synaptosomal preparation. In addition K^+-evoked Ca^{2+} influx was included to assess the viability of each preparation.

2.4 Immunohistochemistry

Protocols for immunohistochemical staining were obtained from Michael Iagallo (Histopathology Service Lab, UBC, personal communication, 1996). Rat tissues (thymus, vas deferens, and brain) were collected, rinsed in PBS, and fixed in 4% paraformaldehyde for 16 h at 4°C. Tissues were then placed in PBS followed by PBS/10% sucrose (as a cryoprotectant) and frozen in OCT compound. Cryostat sections were cut at 8 µm and collected on adhesive coated microscope slides. Slides were then placed in PBS/0.01% Triton X-100, warmed to 37°C for 5

min to remove OCT compound, covered with Sequenza cover plates (Shandon, Pittsburgh, PA) and placed in a humidified Sequenza slide rack. Primary antibodies (anti-P_2X_1, anti-P_2X_2, anti-P_2X_4 and anti-P_2X_7, Alomone Labs, Jerusalem, Israel) were applied and incubated for 16 h at room temperature. Slides were rinsed three times with PBS/Triton X-100. Secondary antibody (Oregon Green 488-labelled goat anti-rabbit, Molecular Probes, Eugene, OR) was applied to sections for 2 h under the same conditions. Following incubation, the slides were washed in three changes of PBS/Triton X-100, and coverslipped using Prolong anti-fade mounting media. A negative procedural control, in which the primary antibody was omitted and replaced with diluent buffer only, was used with each series. After the final PBS rinse, the slides were mounted using Prolong anti-fade mounting media (Molecular Probes) and coverslipped. Slides were visualised using fluorescence microscopy and representative images taken with a Spot 2 digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

2.5 Dye Uptake

Fluorescent dye-uptake procedures were modified from those of Hickman *et al.*, (1994). Three-week-old rat thymuses were dissociated by trypsin digestion. Isolated thymocytes were washed three times in Hank's Balanced Salt Solution (HBSS) + 1 mM Ca^{2+}. Cells were resuspended in HBSS + 1 mM Ca^{2+} with 5 µM YOPRO-1 (Molecular Probes, Eugene, OR) and incubated at 37°C. After a 10 min treatment with 1mM ATP, the cells were washed 3 times, resuspended and an aliquot was applied to a microscope slide and coverslipped. Cell preparations were visualised using fluorescence and brightfield microscopy and representative images were captured using Metamorph (Universal imaging, West Chester, PA). Positive staining cells were counted as a percentage of the total number of cells in each image.

2.6 Molecular and Apoptosis Detection Techniques

2.6.1 Western-Blotting

Rat brain, vas deferens or purified rat synaptosomes were mixed and incubated on ice for 20 min with 300-500 µl 1 x SDS gel loading buffer, containing 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol. Protein concentration was measured using Coomassie Protein Assay Reagent (Pierce, Rockford, IL). The sample was heated in boiling water for 5 min and an equal amount of protein from each sample was electrophoresed on 8% SDS acrylamide gels and electrophoretically transferred to a nitrocellulose membrane. Prestained SDS-Page standards (Bio-Rad, Mississauga, Ontario, Canada) were used to ensure the successful transfer and measure the size of any signal thereafter. The membrane was blocked with PBS containing 0.1% Tween (PBST) and 5% skim milk overnight at 4°C and then washed for 10 min with PBST. This step was repeated an additional three times. The washed membrane was incubated with a 1:300 dilution of P_2X_1, P_2X_2 or P_2X_7 antibody in PBST solution for 90 min, washed again and incubated for 90 min with a peroxidase labelled anti-rabbit antibody (1:3000 dilution, included in ECL-Kit from Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). The membrane was washed again and positive bands were visualised with the enhanced

11

chemiluminescence reagents following the instructions of the manufacturer. P_2X_1 and P_2X_2 antibodies were obtained from Alomone Labs (Jerusalem, Israel) while P_2X_7 antibodies were obtained from two different sources (Alomone Labs, Jerusalem, Israel and Chemicon International, Inc., Temecula, California), with similar results being obtained using both antibody sources.

2.6.2 Genomic DNA Analysis

The detection of degraded DNA products was carried out essentially as described by Herrmann *et al.* (1994), cells were lysed in 50 µl of lysis buffer (50 mM Tris, pH 7.5, 20 mM EDTA, and 1% Nonidet P-40) for 30 sec. After centrifugation at $2,000g$ for 5 min at room temperature, the fragmented DNA in the supernatant was collected. After repeating the centrifugation step once, the supernant was further treated at $56^{\circ}C$ for 2 h with RNase A (5 mg/ml) and SDS (1%), followed by digestion with proteinase K (2.5 mg/ml) at $37^{\circ}C$ for 2 h. DNA was precipitated and electrophoresed in 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and visualized under UV light. A 100-bp DNA ladder (Bio-Rad, Mississauga, Ontario, Canada) was used as a size marker.

2.6.3 TUNEL Reaction

For *in situ* nick end labeling (TUNEL reaction), cells were plated onto 25 mm Thermonox plastic culture coverslips and allowed to grow to subconfluency prior to experimental use. After HD treatment (typically 4 -6 h) the medium was removed and the coverslips were washed twice with PBS, and then fixed with 4% paraformaldehyde for 30 min. The cells were permeabilized for 2 min with 0.1% triton X-100, 0.1% sodium citrate and after air drying, 50 µl of TUNEL reaction mixture (Roche Molecular Biochemicals, Laval, Quebec, Canada) was added so as to cover all cells. The coverslips were incubated at $37^{\circ}C$ in a humidified chamber for 60 min and then rinsed 3 times with PBS prior to analysis by fluorescent microscopy.

2.6.4 Annexin V and Propidium Iodide Staining

In the early phase of apoptosis, phosphatidylserine is translocated from the inner part of the membrane to the outer layer. Fluorescein-conjugated Annexin V specifically binds to the phosphatidylserine on the outer leaflet of the cell membrane. Propidium iodide was used in combination with Annexin V to differentiate apoptotic from necrotic cells. Cells were cultured in 24 well plates to subconfluency prior to experimental use. After HD treatment (typically 4 to 6 h) the treatment medium was removed and the cells washed twice with PBS. The cultures were then treated with 50 µl of Annexin V and propidium iodide staining-solution (Roche Molecular Biochemicals, Laval, Quebec, Canada) for 10 min at room temperature and analysed under a fluoresence microscope. Annexin-positive and propidium iodide-negative cells were defined as apoptotic, whereas necrotic cells were positive for both labels.

2.6.5 Soluble DNA

Soluble DNA was measured by the method described by Cui *et al.* (1994) with

modifications. The cells were grown in 24 well plates and log phase growth cultures were radiolabeled by incubation with [^{3}H]thymidine (1 μCi/ml, Amersham Canada Ltd., Oakville, Ont, Canada) overnight at 37°C in a humidified 5% CO_2 incubator. The medium containing [^{3}H]thymidine was then removed and the cells rinsed once with PBS. After drug treatment (3 -6 h), aliquots of the culture medium (1ml/well, part A) were saved for radioactivity measurement and the cells were then lysed in 0.5 ml TET (10 mM TRIS-HCL Ph 7.5, 2 mM EDTA, 0.2 % Triton X-100) at 4°C for 30 min. The cell-lysate was then centrifuged (22 min, 12,000g) and the resulting supernatant (part B) was removed and counted. The lysate pellet (part C) was solublilized with 1N NaOH (0.2 ml/well) and counted. The experiments were performed in triplicate. Soluble DNA (percentage) was calculated according to the following formula: (Part A CPM + Part B CPM)/ (Part A CPM + Part B CPM + Part C CPM) x 100.

2.6.6 Morphological Observations

Cell cultures were grown to subconfluency in 24 well plates prior to experimentation. After HD treatment (4 - 6 h), the cells were washed with PBS and then stained with 10 μl dye mix (100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS) as previously described (Duke and Cohen, 1992). The cells were then visualized and scored using fluorescent microscopy. A minimum of 200 cells were visualized, recording the incidence of each of the following four cellular states; i) viable cells with normal nuclei (VN; bright green chromatin with organized structure), ii) viable cells with apoptotic nuclei (VA: bright green chromatin which is highly condensed or fragmented), iii) nonviable cells with normal nuclei (NVN; bright orange chromatin with organized structure) and iv) nonviable cells with apoptotic nuclei (NVA; bright orange chromatin which is highly condensed or fragmented). The percentages of apoptotic and necrotic cells were then calculated according to the formula:

% apoptotic cells = (VA+NVA)/(VN+VA+NVN+NVA) x 100.
% necrotic cells= NVN/(VN+VA+NVN+NVA) x 100.

2.6.7 Detection of mRNA for P_2X_1 and P_2X_7 Receptors in Rat Thymocytes, Vas Deferens and Synaptosomes

Total RNA was extracted from rat thymocytes, vas deferens and synaptosomes according to a guanidinium isothiocyanate protocol as previously described (Ghahary *et al.*, 1994). cDNA (RT-cDNA) was made from total RNA by using a cDNA kit (Boehringer Mannheim, Quebec, Canada) and following the manufacturer's instructions. PCR was carried out in an automatic thermal cycler (PTC-2000 peltier thermal cycler, MJ Research, MA). Sense primers with the sequence of 5'-AGAGGCACTACTACAAGCAGAA-3' (for P_2x1) and 5'-AGGAGCCCCTTATCAGCTCT-3' (for P_2X_7), and antisense primers with 5'-GGTAAGGCTGTGGGAAAGA-3' (for P_2X_1) and 5'-CATTGGTGTACTTGTCGTCC-3' (for P_2X_7) were used to amplify the cDNA specific for the P_2X_1 and P_2X_7 receptor. The reaction mixture contained 1.5 M $MgCl_2$, 200 μM of dATP, dCTP, dGTP and dTTP, 20 pmol of P_2X_1 sense and antisense primers or 50 pmol of P_2X_7 sense and antisense primers, 1.5 units Taq DNA

polymerase (Boehringer Mannheim, Quebec, Canada) for P_2X_1 and 2.5 units Taq DNA polymerase for P_2X_7, and 0.25 of the RT-cDNA (5µl) in a final volume of 50 µl. The PCR conditions were 60 sec of denaturing at 94, 60 sec of annealing at 55, and 90 sec of extension at 72 for 35 cycles with an initial denaturing cycle at 94 for 3 min and a final extension cycle at 72 for 5 min. The amplified products were analyzed on a 1.2% agarose (Gibco BRL, Grand Island, NY) gel containing 0.5 µg/ml ethidium bromide. The size of the PCR product was judged by using 100 base pair (bp) DNA molecular weight markers as references (Boehringer Mannheim, Quebec, Canada).

3. RESULTS

3.1 Cellular Toxicity Assays

HD treatment resulted in dose-dependent cytotoxicity in all five of the cell types as measured by the alamarBlue assay. Fig. 1 shows the effects of HD on neurons, keratinocytes, the cell lines J774 and CHO-K1, and on rat thymocyte preparations. The quantitative values for these results are shown in Table 1. Dexamethasone has been included in the thymocyte results since it has been reported to induce apoptosis in thymocytes by a mechanism which may include the upregulation of P_2X_1 mRNA (Chvatchko et al., 1996). The dexamethasone toxicity in thymocytes reported here is similar to previous reports in the literature. However, our initial attempts to demonstrate ATP toxicity in thymocytes have been disappointing. We are planning to begin flow cytometry experiments to identify the presence of apoptosis or necrosis in thymocytes treated with ATP. Ultimately the goal is to observe whether dexamethasone, ATP and HD share common effects on P_2X_1 mRNA.

Table 1.
24 Hour Cellular Toxicity of HD Exposure on Various Cell Types (LC_{50} µM)

Neurons:	
Immature	40.6
Mature	14.5
Keratinocytes*:	
Proliferating	45.1
Confluent	215.4
CHO-K1	
Sub-confluent	204.9
J774	
Sub-confluent	63.4
Thymocytes	5

*Keratinocyte toxicity was assessed after 48 h exposure

3.2 Evidence for Apoptosis and Necrosis in a Variety of Cell Types

The results presented in this section characterize HD induced cell death in a variety of cell types. In general, the results show that HD induces both apoptotic and necrotic cell death in all 4 tissue types described here, as well as in incomplete keratinocyte studies.

3.2.1 J774

J774 cells were sensitive to the cytotoxic effects of HD at approximately 25- 75 µM, the toxicity being somewhat dependent on factors such as cell confluency (Fig. 1). Figs. 2 to 4 show that HD initiated apoptosis at similar concentrations to those which caused cell death as judged from the cytotoxicity assays shown previously (Fig. 1, Hamilton *et al.*, 1999; Sawyer and Hamilton, 1999). In J774 cells stained with acridine orange/ethidium bromide, normal cells appear greenish while apoptotic cells contain a dense yellowish green fluorescence indicative of condensed chromatin. The necrotic cells stain red in colour and are larger than apoptotic cells (Fig. 2). The TUNEL assay (Fig. 3) also shows increasing numbers of apoptotic cells as the concentration of HD increases but doesn't distinguish necrotic from apoptotic cells. Staining with acridine orange/ethidium bromide shows a concentration-related increase in apoptotic cells in response to HD. However, this technique also shows that if a constant incubation time is employed, the proportion of cells that die from necrosis increases to about 50% of the population of dead cells, at the higher concentrations of HD (Fig. 4).

3.2.2 CHO-K1 Cells

CHO-K1 cells were less sensitive to the toxic effects of HD than were J774 cells. However, both cell types underwent the dual processes of apoptosis and necrosis following HD exposure. Fig. 5 shows the response of CHO-K1 cells stained with acridine orange/ethidium bromide. Untreated CHO-K1 cells stained a greenish colour when exposed to acridine orange/ethidium bromide (Fig. 5A). Apoptotic cells are those which contain yellowish dots consisting of condensed chromatin, a hallmark of apoptosis (Fig. 5B,C). The necrotic CHO-K1 cells were somewhat larger than apoptotic cells and are identified as those that stained an orange colour (Fig. 5B, C). Fig. 6 shows cells that have been stained with annexin V and propidium iodide prior to exposure to HD. Again, both types of toxicity, apoptosis and necrosis, are evident using this technique. Further evidence of HD induced apoptosis is shown in Fig. 7 and the quantitative data is summarised in Table 2.

Table 2
Quantification of Apoptotic CHO-K1 Cells Treated with Different Concentrations of
Sulphur Mustard (HD) by the TUNEL Reaction (Mean ± sd; n=5)

HD (µM)	200	400	600
Apoptotic CHO-K1 cells (% of Total)	8.9 ± 1.6	27.8 ± 14.4	50.8 ± 9.1

The presence of HD induced DNA ladders is clearly evident in Fig. 8. In agreement with the results of the TUNEL reaction that also demonstrated the presence of apoptosis, a 6 h exposure to HD produces DNA fragmentation, a phenomenon generally accepted as indicative of apoptosis. As was the case with J774 cells, there was clear evidence that the number of apoptotic cells observed began to increase coincident with the initiation of cell death as measured by cytotoxicity assays (Fig. 1). It was also evident that the proportion of apoptotic cells increased in a dose-dependent manner with increasing HD concentration (Table 2).

3.2.3 Chick Neurons

Cultured chick neurons were also included in our studies examining the effects of HD on neuronal preparations from the CNS. In addition to its vesicant effects, HD can also have profound systemic effects at high exposures, causing convulsions and other effects on the CNS (Papirmeister et al., 1991). Long term central effects of HD have also been reported in soldiers exposed to HD from the Iran- Iraq war. The studies carried out to date indicate that these neurons are very sensitive to HD induced toxicity and as in all other cell types examined, undergo apoptosis and necrosis. Fig. 1 shows that HD exposure results in a fairly steep concentration response curve for neurons but at the highest concentration used (90 µM) in these experiments, it was not possible to determine whether the proportion of apoptotic cells had reached a stable proportion of the dead cells. Fig. 9 shows representative examples of the increase in the number of apoptotic cells, detected using the TUNEL reaction, after HD exposure. Table 3 quantitates these experiments and shows that the number of apoptotic cells doubles over the concentration range tested.

Table 3.
Quantification by the TUNEL Reaction of Apoptotic Chick
Neurons Following Exposure to HD for 6 Hours.

HD (µM)	0	25	40	65	90
Apoptotic Cells (% Total)	9.9	14	17.1	19.4	20.2

The experiments to examine the presence of necrotic neuronal cells in cultures exposed to HD and the presence of DNA ladders from these cells have not yet been completed.

3.2.4 Immature Thymocytes

The toxicity of HD in thymocytes was somewhat greater than in other cell types. Fig. 1 shows that HD toxicity in thymocytes was evident at concentrations as low as 1 µM. Dexamethasone has been shown to be a potent inducer of apoptosis and its toxic effects have been suggested to occur through the up-regulation of the P_2X_1 receptor (Chvatchko *et al.*, 1996). It is evident that dexamethasone is more toxic than HD in this tissue type. Studies to identify apoptosis in thymocytes exposed to either HD or dexamethasone using the methods described are incomplete and have not been included here. Work is presently underway to examine whether HD, ATP or dexamethasone produce an up-regulation of P_2X_1 mRNA in these cells coincident with the initiation of apoptosis. To this end, the results presented below in Section 3.4, were designed to positively identify the P_2X receptor subtypes in this cell type.

3.3 Intracellular Calcium Levels in Cells Exposed to HD

The results of our studies on the alteration of calcium homeostasis in keratinocytes exposed to HD are found in Annex A (Sawyer and Hamilton, 1999). Briefly, the results indicate that cells exposed to HD respond with a rapid, although modest increase in intracellular calcium levels. HD still caused a rise in $[Ca^{2+}]_{INT}$, even in the absence of extracellular calcium. Similarly, no modulation of HD induced toxicity was observed in keratinocyte cultures in which calcium influx was inhibited by reducing extracellular calcium levels to zero, or following the depletion of the intracellular calcium stores with thapsigargan. This suggests that the increased calcium levels observed were not directly responsible for the subsequent toxicity, but might rather have been the result of the toxic insult. We show here, in both J774 cells and CHO-K1 cells, that HD exposure resulted in a rapid increase in $[Ca^{2+}]_{INT}$ (Fig. 10). These studies are not yet complete and further work is currently underway in order to examine whether altering calcium function, for example by utilising calcium blocking agents, such as ω-conotoxins, dihydropyridines, ryanodine, etc, will directly affect the toxicity of HD or whether the HD induced calcium elevation is the result of some membrane alteration (*e.g.* fluidization) induced by HD.

3.4 Identification of ATP Receptors in Various Cell Types Responsive to HD

3.4.1 P_2X Receptors in Synaptosomes Mediating Ca^{2+} Influx

Experiments were carried out to determine possible interactions of ATP with purinergic receptors associated with neurons. Fig. 11 provides immunohistochemical evidence of P_2X_1 and P_2X_7 receptors in whole rat brain but fails to localize them. Receptor localisation is important since the presence of P_2X_7 receptors on non-neuronal microglial cells has previously been reported. We therefore prepared rat brain synaptosomes, or "pinched off nerve endings", the site of active neurotransmitter release, from rat brain cortices. These synaptosomes were exposed to

17

ATP and benzyl-ATP (BzATP), which is a selective P_2X_7 agonist, for varying periods of time in order to determine the optimal incubation time for the structure activity studies described below. The results presented in Fig. 12 show that calcium influx increased for up to about 60 sec. This time was subsequently chosen as the time used in all further experiments. The results in Fig. 13 indicate that BzATP was considerably more active in the induction of calcium influx than was ATP. A structure activity profile with a variety of ATP analogues was carried out to provide pharmacological evidence for the identification of the type of P_2 receptor responsible for the influx which was observed. The results in Fig. 14 show the following order of potency for a variety of analogues on calcium influx: BzATP > ATP > 2-Me-thio-ATP-S > α,β-Me-ATP > β,γ-L-ATP, a profile which is consistent with the presence of the P_2X_7 receptor (Wiley *et al.*, 1996). Further confirmation of the presence of this receptor was gained following positive identification of the P_2X_7 receptor with the use of Western blotting using anti P_2X_7 antibodies from two commercial sources. Figs. 15 and 16 show identical results in representative blots obtained using both anti-P_2X_7-antibodies. Despite the presence of the cytolytic P_2X_7 receptor in rat brain synaptosomal preparations, calcium influx was not stimulated by HD nor did HD potentiate Ca^{2+} influx evoked by ATP (Fig. 17), at least at the 15 min HD incubation time used in these experiments. Similar results (*i.e.* no effect) were obtained with concentrations of HD up to 1 mM.

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3.4.2 P_2X Receptors in Vas Deferens

Initial studies were carried out to confirm the presence of the P_2X_1 type of P_2 receptor in the rat vas deferens smooth muscle preparation. This was important since we plan to carry out studies in the future to identify changes in the regulation of this receptor protein in response to HD and to other inducers of cellular apoptosis, such as dexamethasone. The results of PCR, which was performed on RNA extracted from the vas deferens cells are shown in Fig. 18. The P_2X_1 band is clearly present but there is also a 693 bp staining which suggests the presence of the P_2X_7 receptor protein that has not previously been identified in the vas deferens. We attempted to confirm this result using immunohistochemical staining. The results, shown in Fig. 19, support this finding and appear to confirm the presence of both P_2X_1 and P_2X_7 receptors in this smooth muscle tissue. The finding of an additional P_2X receptor subtype is important in the explanation of some of the structure activity relationships previously reported in the literature (Wiley *et al.*, 1996) which were based on the assumption that only P_2X_1 type receptors were present.

3.4.3 P_2X Receptors in Thymocytes

The attempts to demonstrate apoptosis and necrosis from ATP and HD challenges are inconclusive to date. Although HD readily causes cytoxicity in thymocytes (Fig. 1), to this date it is not clear whether cell death is apoptotic or necrotic in nature. These studies are ungoing.

We undertook experiments to identify the type of P_2 receptors that exist in thymocytes. Fig. 20 shows RT-PCR RNA extract stained with ethidium bromide. The 693 bp is specific for P_2X_7 and the 434 bp is specific for P_2X_1. These results are supported in Fig. 21 that shows immunohistochemical detection of these two ATP receptors in thin sections of rat thymus. Further evidence of the functional expression of ATP sensitive P_2X receptors is shown by the ATP

mediated uptake of the fluorescent dye YOPRO-1 (Fig. 22). In this figure the left-hand panels show the brightfield image of the thymocytes and in the right hand panels the cells that were fluorescently labelled by YOPRO-1. The dose-response relationship between dye uptake and ATP concentration is shown in Fig. 23. One of the main problems we have encountered to date consists of our inability to demonstrate apoptosis in thymocytes exposed to ATP. Although ATP induced apoptosis in thymocytes has been widely reported in the literature (Zheng *et al.,* 1991; Freedman *et al.,* 1999) we have not been able to demonstrate ATP induced cell death using alamarBlue, an assay indicative of metabolic viability. This particular method indicates that the cells are still alive, indeed thriving, at concentrations of ATP up to 12.5 mM following 24 hours of exposure. We are currently carrying out experiments with specific ATP receptor blocking agents in order to identify the ATP receptor responsible for the dye (YOPRO-1) uptake. Also we are now initiating the use of flow cytometry in an attempt to repeat the results of others with respect to ATP induced apoptosis in thymocytes.

4. DISCUSSION

HD was toxic to all cell types that were exposed to it. This was not surprising since the acute toxicity of HD *in vivo* and *in vitro* has been widely reported in the literature. It is known from animal experiments and certainly from accidental and intentional human exposures in military conflicts (for reviews see Dacre and Goldman, 1996; Willems, 1989), that HD affects not only the obvious targets, which are the skin and eyes, but a wide variety of other tissues, including the GI, respiratory and immune systems and the brain (Papirmeister *et al.,* 1991). It has also been reported that HD and HD analogues can cause cytotoxicity through apoptotic cell death (Dabrowska *et al.,* 1996; Hur *et al.,*1998; Rosenthal *et al.,* 1998). The present studies extend these findings and show clearly and conclusively, using a wide range of tests, that exposure to HD causes both apoptosis and necrosis in cells obtained from the immune system, the skin, and the CNS. Not all cells were equally sensitive to the cytotoxic effects of HD, but the signs of apoptosis began to appear at concentrations of HD which were found to induce cytotoxic effects in assay systems such as alamarBlue. In all cell types, increasing concentrations of HD resulted in a larger proportion of cells being necrotic, but it is not presently clear whether these cells progressed through an apoptotic stage during the degenerative process.

4. 1 HD Induced Apoptosis/Necrosis in Various Cell Types

4.1.2 Thymocytes

Thymocytes, and the response of thymocytes to HD, were chosen for study for several related reasons. Since we have proposed that HD and ATP might share some common sites for the induction of apoptosis, and because dexamethasone also might act at a similar site, this cell type was chosen to compare ATP, dexamethasone and HD induced cytotoxicity. Thymocytes have been extensively studied with respect to two very important aspects of our work: i) their response to ATP and dexamethasone and ii) the ATP receptors responsible for the cytotoxicity of these two

19

compounds have been well studied. The literature clearly suggests that thymocytes exposed to ATP undergo apoptosis and it has been postulated that the apoptotic events are initiated by the stimulation of the membrane P_2X_1 receptor (Zheng *et al.*, 1991, Pizzo *et al.*, 1991) and also by the up-regulation of the P_2X_1 receptor mRNA (Chvatcho *et al.*, 1996). Furthermore, ATP induced cytotoxicity can be predictably modified by manipulating receptor and agonist (ATP) activity. For example, reducing the activity of ATP at the P_2X_1 receptors with appropriate blocking agents such as suramin or by decreasing ATP levels by increasing ATP metabolism, has been reported to inhibit the apoptotic cell death produced by ATP and its analogues (Chvatcko *et al.*, 1996; Freedman *et al.*, 1999). Calcium influx has also been suggested to play a critical role in the initiation of apoptotic events in general (Dowd, 1995; Orrenius *et al.*, 1989). It has been postulated that an initial event in the induction of ATP induced apoptosis involves the ATP induced influx of calcium through the activation of an ATP receptor (Nagy *et al.*, 1995; McConkey *et al.*, 1989; Ross *et al.*, 1997), although no clear cut cause and effect relationship exists between calcium influx and cell death. The results presented here reveal that HD causes a concentration and time dependent apoptosis (and necrosis) in thymocytes determined by several definitive assays. We are currently attempting to demonstrate whether HD causes up-regulation of P_2X_1 mRNA in thymocytes undergoing apoptosis, using dexamethasone as a positive control. The data presented here at this time, is consistent with the suggestion that ATP and HD may act at common sites to produce apoptosis in these cells. However, contrary to results reported by others (Pizzo *et al.*, 1991; Zheng *et al.*, 1991; Freedman *et al.*, 1999) we have been unable to demonstrate apoptosis in thymocytes caused by exposure to ATP. We have also conducted experiments in which thymocytes were exposed to ATP and its analogues at the same time as HD, and others where the addition of ATP blocking agents prior to HD exposure, did not alter HD toxicity. These preliminary results argue against the interaction of HD and ATP in the production of apoptosis in thymocytes.

4.1.3 J774 Cells

J774 cells are a macrophage derived cell line, which like thymocytes, have also been reported to undergo cell death on exposure to external ATP (Murgia *et al.*, 1992; Steinberg *et al.*, 1987; Zambon *et al.*, 1994; Coutinho-Silva and Persechini, 1997). This cell type therefore also provides a useful tool with which to examine HD, if HD induced toxicity is indeed mediated by ATP receptors. Moreover, as is the case with thymocytes, ATP induced cell death in J774 cells also appears to be preceded by an increase in intracellular calcium levels which may occur through activation of the ATP ionic pore in this tissue, the P_2X_7 receptor. In addition to the ability of this receptor to control calcium translocation, stimulation of the P_2X_7 receptors is followed, in turn, by the entrance of larger molecular weight species through a second "pore" also associated with P_2X_7 stimulation (Coutinho-Silva and Persechini, 1997; Murgia *et al.*, 1992). In this respect J774 cells differ from thymocytes in that they contain the P_2X_7, but not the P_2X_1 type of receptor. However, while P_2X_7 receptors also mediate cell death, it is not necessarily apoptotic in nature (for review see DiVirgilio *et al.*, 1998). Rather, it appears that exposure to ATP and its analogues, leads to calcium elevation and subsequently, the entry of larger molecular weight substances through the pore associated with this receptor. These larger molecules cause cell disruption through what has

been referred to as colloidal-osmotic lysis rather than apoptosis (Murgia *et al.,* 1992; Zambon *et al.,* 1994; Coutinho-Silva and Persechini, 1997). Nevertheless, our results are completely consistant with the ability of HD to induce concentration-related apoptosis in this tissue as measured by DNA fragmentation, a positive TUNEL assay and staining with ethidium bromide. These results clearly demonstrate that although P_2X_1 ATP receptors may play a role in the initiation of apoptosis in a number of cell types, other types of cell death may be induced in cells in which these receptors are absent. We are currently exploring the possibility of obtaining mutant J774 cells that lack the P_2X_7 receptor. Obtaining these cells will allow the exploration of the possibility that it may play a role in the non-apoptotic mechanisms involved in HD induced cell death (Zambon *et al.,* 1994). However, initial studies in which oxidized ATP, a specific inhibitor of the P_2X_7 ATP receptor, was incubated with these cells failed to reveal alteration of the toxic effects of subsequent HD exposure.

4.1.3 CHO-K1 Cells

One reason why we chose to study the effect of HD on CHO-K1 cells was the fact that these cells appeared not to contain P_2X_7 receptors. In addition, these cells do not express a variety of receptor sub types that make them useful for recombinant receptor expression for future planned work. Nevertheless, our studies indicated that these cells aparently express the P_2X_7 receptor and although ATP was cytotoxic in cultures of these cells, it produced cell lysis (presumably necrosis) rather than apoptosis. Our results also showed that HD caused a concentration dependent cell death that was divided into cells undergoing apoptosis and those displaying necrosis. These studies offer good evidence therefore that HD certainly may induce apoptosis in the absence of P_2X_1 receptors. It was also interesting to note that these cells, when exposed to HD, became permeable to calcium, although at the present time we are not able to suggest any cause effect relationship between HD toxicity and calcium levels. These cells will be useful in our attempts to relate P_2 receptor activity with cell death since the P_2X_7 receptors in this tissue can be inhibited by specific receptor blocking compounds prior to exposure to ATP. These experiments are currently underway.

4.2 Intracellular Free Calcium Levels Following HD Exposure

Recently we have presented evidence indicating that exposure of keratinocytes to HD resulted in a rise in intracellular free calcium levels (Hamilton *et al.,* 1999). However, additional work (Sawyer and Hamilton, 1999; Annex A) has shown that depletion of intracellular calcium from keratinocytes with thapsigargan, or incubation of the keratinocytes in the absence of external calcium, failed to alter the toxicity of subsequent exposure to HD. These studies therefore failed to establish a cause effect relationship between elevated intracellular calcium levels and cell death, at least in keratinocytes.

Our work with J774 and CHO-K1 cells have clearly shown the induction of apoptosis by HD. As was the case in keratinocytes, calcium concentrations began to rise in both cell types shortly after exposure to concentrations of HD sufficient to induce apoptosis. Increased cellular calcium levels have been implicated as a possible triggering event in apoptosis development (and

21

perhaps necrosis as well) in a wide variety of cells (Dowd, 1995; Orrenius *et al.,* 1989). Previous hypotheses concerning mechanisms of HD induced cytotoxicity, including our own (Papirmeister *et al.,* 1991, Lundy *et al.,* 1998), have implicated elevations of intracellular calcium in the proposed toxic cascade following HD exposure. The elevation of intracellular calcium levels may be responsible for the activation of various intracellular proteases and endonucleases. However, to this date no definitive relationship between HD toxicity and an increase in calcium levels has been elucidated. Attempts are on going to determine the validity of the suggestion of a cause effect relationship between HD induced cell death and the elevation of intracellular calcium levels in J774 and CHO-K1 cells. The results to date are consistent with the suggestion that HD exposure results in a rapid and significant elevation of calcium in these two additional cell types at least, and suggest strongly that elevation of intracellular calcium levels is a fairly consistent event following HD exposure. Experiments are currently underway to determine whether HD toxicity can be affected or indeed, whether HD induced apoptosis can be manipulated by inhibiting the rise in calcium levels following HD exposure. We will attempt to manipulate calcium levels in the traditional ways (thapsigargan) and also by exposing the cells to inhibitors of the P_2X_7 receptor (which is also an ATP stimulated calcium channel) found in both of these cell types. These future experiments will reveal whether P_2X_7 receptors play any role per se in HD induced toxicity in these two cell types, or whether the cellular rise in calcium levels following HD exposure is the result of activation of this ionic membrane pore. Toxicity studies will also be carried out after intracellular calcium manipulation in the hope of establishing whether the rise in calcium levels already observed, plays a significant role in the initiation of cell death, or whether they are the result rather than a cause of some toxic mechanism initiated by HD.

4.3 Morphology of Vas Deferens Following Stimulation of P_2X_1 Receptors

This discussion deals with our attempts to observe morphological changes in the whole vas deferens muscle after adding ATP or HD to the solution bathing it. Our original premise suggested that ATP and HD, two cytotoxic compounds, might act or partially act, through the P_2X_1 type ATP receptor in the vas deferens. This proposal was put forth because HD markedly potentiated ATP responses in this tissue, presumably through some effect on the P_2X_1 receptor. It has been proposed that this receptor, when stimulated appropriately by an ATP agonist, might also initiate apoptosis and that the initial event might be the influx of calcium through the ionotropic P_2X_1 receptor (see discussion above). Therefore, it was reasoned that the addition of ATP to the buffer bathing the vas deferens should cause the induction of apoptosis and further, that the potentiation of the ATP-induced contractile effects of this muscle by HD at this receptor, would induce more severe apoptosis or would create the lesion more rapidly. Studies were therefore carried out that were designed to relate observable signs of the stimulation of the P_2X_1 receptor (muscle contraction), with signs of the development of apoptotic events from exposed, then fixed, tissue strips. This was carried out by stimulating the muscle to contract either through the electrically induced release of ATP from adrenergic nerves or as the result of addition of exogenous ATP. These experiments were carried out with ATP acting as the lone agonist and also in the presence of low concentrations of HD, which markedly potentiate the ATP P_2X_1 receptor response, i.e. tissue contraction. After exposure of the muscle for pre-determined time

intervals, tissues were removed from the organ bath, rinsed and then fixed for subsequent histological examination. Although it is now clear that HD potently initiates apoptosis in a broad phylogenetic spectrum of cells, the results obtained in the present studies failed to demonstrate cell death in the fixed vas tissues as determined morphologically (results not shown). We believe that since HD causes apoptosis in a variety of different cell types, it is likely that it will also result in the development of apoptosis in vas deferens cells. It is suggested that the failure of HD and perhaps ATP to initiate apoptosis in the vas tissue was almost certainly due to experimental conditions which were not sufficiently rigorous to initiate the cytotoxic process. For example, in the present studies, whole vas deferens strips were exposed to a relatively low (100 μM) HD concentration for only 5 min. Initiation of apoptosis in other cell types required, not necessarily higher concentrations of HD in all cell types, but certainly much longer exposure times. These experiments will be repeated employing longer exposure times and higher concentrations of HD with ATP. It should also be pointed out that studies have been initiated, so far without success, to separate individual vas cells from the whole tissue, in order that studies can be carried out in cell culture as well as in the whole viable tissue.

4.3.1 Central Nervous System Synaptosomes.

The results of our innervated smooth muscle work suggested the possibility of an interaction of ATP and HD at ATP receptors, or on HD induced neurotransmitter release. This interaction could conceivably take the form of an interaction of the two compounds at one of two sites; either through direct interaction at the P_2X post synaptic receptor or at presynaptic receptors through a potentiation of calcium influx, thereby increasing neurotransmitter release. In order to test the latter hypothesis, experiments were carried out to pharmacologically characterize the P_2X receptors in presynaptic nerve terminals from rat brain. These P_2 receptors are critical in nervous function since they function as ion channels supplying the calcium necessary for neurotransmitter release in response to ATP. Since all of the experiments were carried out in the presence of voltage sensitive calcium channel blocking drugs (ω-conotoxin MVIIC), the studies not only pharmacologically identified the presence of presynaptic ATP receptors using structure activity profiles, but also demonstrated clearly that they are unrelated to voltage sensitive calcium channels. Since this finding was both novel and controversial, we therefore carried out further studies in order to positively identify the presence of P_2X_7 receptors in this tissue. Western blotting clearly showed the presence of P_2X_7 receptors on presynaptic terminals. However when the calcium influx studies were carried out in the presence of HD there was no additional increase in calcium influx beyond that resulting from ATP exposure alone.

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstration of HD induced toxicity in a variety of different cell types, including cells from skin, brain and the immune system.
2. Conclusive evidence of both apoptosis and necrosis following sulphur mustard challenges in every cell type examined.
3. Demonstration that sulphur mustard induced calcium influx in keratinocytes is not causally related to cytotoxicity in this cell type.
4. Demonstration of sulphur mustard induced calcium influx in other cell types (J744, CHO-K1). Possible cause effect relationships are currently under investigation.
5. Identification of heretofore unrecognized P_2 purinoceptor subtypes in brain, vas deferens and immature thymocytes.
6. Evidence that ATP induced cytotoxicity/apoptosis in certain cell types may be artefactual.

REPORTABLE OUTCOMES

1. Sawyer, T.W. and Hamilton, M.G. (1999). Effect of intracellular calcium modulation on sulphur mustard cytotoxicity in cultured human neonatal keratinocytes. *Toxicol. In Vitro* (In Press).

CONCLUSIONS

1. Sulphur mustard induced increases in intracellular calcium is not related to cytotoxicity in keratinocytes. However, this lack of cause effect relationship has yet to be demonstrated in other cell types.

2. Sulphur mustard induces apoptotic cell death in all cell types examined thus far. This appears to be a general phenomenon.

3. Apoptosis caused by sulphur mustard is not calcium dependent.

4. The reported cytotoxic activity of ATP in thymocytes does not occur in our studies.

5. There are P_2X_7 receptors in the central nervous system. This is an important, novel finding.

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ANNEX A

Sawyer, T.W and Hamilton, M.G. (1999). Effect of intracellular calcium modulation on sulphur mustard cytotoxicity. *Toxicol. In Vitro* (**In Press**).

Effect of Intracellular Calcium Modulation on Sulphur Mustard Cytotoxicity

in Cultured Human Neonatal Keratinocytes

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Abbreviations: BAPTA-AM = 1,2-bis (O-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid; HD

= bis 2-(chloroethyl) sulphide, sulphur mustard

ABSTRACT

Previous studies in human skin keratinocyte cultures have shown that sulphur mustard (HD) induces an immediate and irreversible increase in internal free calcium levels that was independent of external calcium concentrations. These findings suggested a role for calcium in the etiology of HD-induced cell death and that modulation of intracellular calcium concentrations may assist in providing protection against this agent. In the current work, actively proliferating and confluent cultures of first passage neonatal human skin keratinocytes were used to assess the effect of altered intra- and extracellular calcium levels on HD toxicity. Treatment of cultures with the endoplasmic reticulum calcium ATPase inhibitor; thapsigargin, or the calcium chelator BAPTA-AM, which reduce HD-induced elevation of intracellular free calcium, did not modulate the toxicity of HD. Furthermore, alteration of external calcium concentrations during these same experiments failed to elicit any change in the viability of HD-exposed cells. Treatment of confluent cultures with ionomycin at either low (100 μM) or high (1.2 mM) external calcium concentrations also failed to modulate the toxicity of HD in any way. It appears, that in neonatal human skin keratinocytes in culture, HD-induced intracellular calcium perturbation does not play a major role in HD-induced cytotoxicity.

2

INTRODUCTION

Sulphur mustard (HD, bis 2-(chloroethyl) sulphide) is a stong alkylating agent which, in addition to causing extensive DNA damage, is also capable of exerting deleterious effects in a variety of tissues including skin vesication, eye and respiratory tract injuries and systemic intoxication (Dacre and Goldman, 1996; National Academy Press, 1993; Papirmeister et al., 1991; Smith and Dunn, 1991; Warthin and Weller, 1919). Although this compound has attracted a great deal of research attention due to its continued use as a chemical warfare agent, its mechanism of toxic action is as yet unknown. A number of hypotheses have been put forward to explain the toxicity of HD, most recently several that propose that HD-induced cell death is either calcium-dependent, or is the result of disrupted intracellular calcium homeostasis. Although several different laboratories have shown that HD induces a rise in intracellular calcium in cultured cells (Hamilton et al., 1998; Hua et al., 1993; Mol, 1994; Mol and Smith, 1996; Ray et al., 1994; 1995) or in whole tissue (Lundy et al., 1998), the level, and the importance of this elevation in calcium has been debated. Recent work in our laboratories has shown that secondary cultures of confluent human skin keratinocytes exposed to HD, rapidly ($\leq$ 2 min) exhibited a concentration-dependent, irreversible increase in intracellular calcium that appeared to originate from intracellular stores. Removal of the HD from the cultures following a 2 min exposure did not change these findings significantly and the rapidity of the calcium response paralleled the cytotoxicity of HD, which was expressed maximally at 48 h after only a few minutes of actual HD exposure (Hamilton et al., 1998).

An extensive body of work exists that demonstrates that calcium is widely known to mediate the toxicity of a variety of drugs through activation of lytic enzymes and/or calcium-

3

dependent receptors. Furthermore, prevention of induced calcium elevation has been shown in many cases to be protective. Previous work showing that HD elevates intracellular calcium suggests that drug regimens aimed at modulating these calcium stores may therefore be of utility in ameliorating HD toxicity.

Normal mouse and human skin keratinocyte culture have been shown to be dynamic systems in which the differentiative state of the cells is intimately related to the intracellular calcium environment, which in turn is dependent on both the external calcium concentration in the medium, as well as the confluency of the cultures themselves (Hennings *et a*l., 1980; Kruszewski *et al.*, 1991; Pillai *et al.*, 1990; Sharpe *et al.*, 1989; Yuspa *et al.*, 1989). We therefore examined the effects of treatments which modulate both external and internal calcium concentrations on HD-induced toxicity in both actively proliferating and confluent cultures of neonatal human skin keratinocytes.

METHODS

Materials   Trypsin, foetal calf serum and Fungizone were purchased from Flow Laboratories (Mississauga, Ontario, Canada). AlamarBlue™ was acquired from AccuMed International Inc. (Westlake, OH). Keratinocyte Serum Free Medium (KSFM) was obtained from Gibco BRL, Grand Island, NY) and dispase from Collaborative Research (Bedford, MA). Pluronic F-127, 1,2-bis (O-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) and Fura-2 AM were supplied by Molecular Probes Inc. (Eugene, OR), while thapsigargin and ionomycin were obtained from Research Biochemicals International (Natick, MA). Sulphur mustard was prepared by the Hazard Avoidance Section, Defence Research Establishment Suffield at greater than 99% purity.

Cell Culture   Primary cultures of human skin keratinocytes were prepared from neonatal foreskins. Tissue was obtained on the day of circumcision and incubated at 4 °C for 24 h in 25 U/mL dispase. The epidermis was removed and the cells segregated by a further 5 min incubation in 0.25% trypsin at 37 °C. The trypsin was deactivated by the addition of 20 % serum in medium and the cell suspension was centrifuged. The cell pellet was resuspended in KSFM and filtered through 70 µM nylon mesh. 75 cm^2 flasks were seeded at 5 x 10^5 cells/8 mL KSFM supplemented with gentamicin (50 µg/mL) and Fungizone (0.25 µg/mL) and incubated in a 37 °C humidified incubator in a 5% CO_2/95% air atmosphere. Cultures were re-fed every 2 - 4 days, as required. First passage cultures were seeded from log growth primary cultures at a density of 1000 cells/well in 96-well Costar multiwell plates or onto 13 x 14 mm glass coverslips (0.15 mm thick, Biophysic Technologies, Inc.) in 35 mm culture dishes at 30,000 cells/dish.

Chemical Treatment and Cytotoxicity Studies On the day of chemical treatment the cultures (proliferating; 3-4 days *in vitro*, confluent; 7 - 9 days *in vitro*) were treated with freshly prepared treatment medium so that the desired final HD concentration was reached at 0.25% ethanol (v/v). The viability of HD-exposed cultures was determined at 48 h. In experiments which assessed the effects of BAPTA-AM, ionomycin or thapsigargin on HD toxicity, the compounds were dissolved in ethanol or DMSO (BAPTA-AM) and administered to the cultures 1 h prior to HD treatment. In studies where external calcium was also varied, 1 h before drug treatment, the cultures were aspirated, rinsed twice with 200 μL of phosphate buffered saline and then re-fed with calcium-free medium that had been adjusted to the desired calcium concentration with calcium chloride. In these experiments FURA 2 was not used to quantitate the external calcium and therefore these values represent the *nominal* calcium concentrations. In all experiments, test drugs were left in the cultures for the full 48 h test period. The test drug vehicles (ionomycin; 0.046 % ethanol, thapsigargin; 0.1 % ethanol, BAPTA-AM; 0.15 %) had no effect on the viability of the cultures, even in combination with the ethanol used as the HD vehicle. To assess cytotoxicity, alamarBlue™ was added (10%, v/v) and the cultures were allowed to incubate with the dye for the last 2 h of the treatment time period. This assay is based on the reduction of a dye by viable cells to a coloured species which can be measured by absorbence or fluorescence, and has also been found to yield similar results compared to a number of more commonly used dyes and indicators (Fields and Lancaster, 1993). The absorbances (570 nm - 600 nm) were then read on a Thermomax titerplate reader (Molecular Devices, Sunnyvale, CA). Median lethal concentration (LC$_{50}$) values were determined graphically from experiments utilizing 6 wells per data point and percent protection was expressed as the LC$_{50}$ of drug-pretreated, HD-treated

cultures divided by the LC_{50} of vehicle-pretreated, HD-treated cultures x 100. All values represent data obtained from at least 3 separate experiments.

Cytosolic Calcium Determination Studies to assess the effect of HD on FURA fluorescence were performed using the cell impermeant pentapotassium salt of Fura-2 (0.4 uM) in high (1mM, pH 7.4) and relatively low (225 nM, pH 7.4) Ca^{2+} buffer solutions (Molecular Probes, OR). Fluorescence of Fura-2 was monitored with a Delta-Scan 1 dual excitation/emission fluorometer with front-surface optics sample compartments (model D105; Photon Technology International Inc., Princeton, NJ). The output from the xenon lamp was directed to two excitation monchromators with wavelengths set a 340 and 380 nm (5 nm bandpass), respectively, using a chopper wheel. Output from the excitation monochromators was focused on a 1 cm^2 quartz cuvette by a fibre-optic cable and the fluorescence collected through an emission monochromator at 510 nm (5 nm bandpass) using a photomultiplier tube.

Experiments were performed after loading the cells with the membrane permeant form (Fura-2 AM) of the calcium sensitive dye Fura-2 (Grynkiewicz *et al.*, 1985, McDonough and Button, 1989). The culture medium was removed from cells grown on coverslips and rinsed three times with HEPES buffered Hank's balanced salt solution (pH 7.4, 1 mM nominal Ca^{2+} concentration. The cells were then incubated in the dark with 3 µM Fura-2 AM in DMSO/pluronic-127 for 45 min at room temperature. After incubation, the buffer was changed to buffer containing no Fura-2 AM and allowed to equilibrate for least 30 min prior to recording fluorescence signals. The coverslip was placed in a quartz cuvette so that the cells faced the excitation light path and the fluorescent emission passed through the coverslip before entering

the emission monochromator. Measurements were made in 2.4 mL buffer with continuous

stirring in a temperature-controlled cuvette holder at 37 °C. HD was added to the cuvettes and

the signal re-acquired after a delay of 1 min to eliminate an artefact due to the dissolution of the

HD in the buffer. Signals were background subtracted using Fura-2 free cells for

autofluorescence and, where appropriate, solvent addition.

RESULTS

Figure 1 shows the toxicity of HD in both proliferating and confluent keratinocyte cultures. Proliferating cultures were extremely sensitive to HD toxicity with an LC_{50} of 56 μM +/- 23 μM (mean +/- SD, $n = 3$). Sulphur mustard was 5 - 6 times less toxic in confluent cultures with an LC_{50} of 300 μM +/- 47 μM (mean +/- SD, $n = 3$). The vehicle (0.25% ethanol) had no discernible effect on the viability of the cultures.

BAPTA-AM and thapsigargin were much less toxic in confluent cultures than in proliferating cultures (Fig. 2a, b). In confluent cultures no toxicity was apparent with thapsigargin at the concentrations used (1.0 - 10.0 μM) while BAPTA-AM was non-toxic at concentrations up to 25 μM. In contrast, both compounds reduced the viability of proliferating cultures in a concentration-dependent fashion. In proliferating and confluent cultures that were treated with these compounds prior to HD exposure, no modulation of toxicity was noted compared to sham-pretreated, HD-exposed cultures (Fig. 2c, d).

In studies which examined the effect of extracellular calcium on cell viability, confluent cultures were much less sensitive to changes in external calcium concentration (Fig. 3). Cultures were grown in KSFM (90 μM calcium) and then changed to medium adjusted to various calcium concentrations at or below 100 μM calcium. When compared to the cells cultured in maximal calcium concentrations, the viability of confluent cultures did not change after 48 h, even at a nominal zero calcium concentration. In contrast, proliferating cultures were very sensitive to external calcium concentrations in this range and the viability of these cells declined rapidly below 75 μM calcium.

Non-toxic concentrations of BAPTA-AM (25 μM) and thapsigargin (10 μM), in

9

combination with varying concentrations of external calcium were examined for their effect on the toxicity of HD in confluent cultures. No treatment, or combination of treatments was found to modulate the toxicity of HD (Fig. 4).

After 48 h of continual exposure, ionomycin was slightly more toxic in cells that were actively proliferating than in confluent cultures (Fig. 5a). The effects of ionomycin on the toxicity of HD were examined only in confluent cultures adjusted to either low (100 μM) or high (1.2 mM) calcium (Fig. 5b). The cells were grown in normal culture medium and then changed into low calcium or high calcium medium. After 1 h the cultures were incubated with ionomycin for an additional 1 h prior to HD treatment. This compound had no effect on HD toxicity at either calcium concentration in confluent cultures.

Figure 6a,b show the effect of various chemical treatments on intracellular calcium levels as measured by Fura-2. Sulphur mustard (1.0 mM) caused a rapid and sustained elevation of intracellular calcium levels (Fig. 6a), while thapsigargin (5.0 μM) induced a transient elevation of calcium that eventually returned back to background levels. Ionomycin (10 μM) caused an immediate elevation of the calcium signal which was instantly quenched by the addition of the calcium chelator BAPTA (25 mM). Lower concentrations of ionomycin (0.1 -1.0 μM) also caused elevations in the calcium signal, albeit at a slower rate and not to the extent that 10 μM ionomycin did (data not shown). The non-ester form of BAPTA-AM was used since BAPTA-AM quenches the calcium signal. As reported earlier (Hamilton et al., 1998), HD by itself neither affected FURA 2 fluorescence nor exhibited any fluorescence of its own in the excitation/emission ranges used in these experiments.

10

DISCUSSION

A variety of hypotheses have been advanced over the last several decades in efforts to explain the vesicant action of HD (reviewed in Papirmeister *et al.*, 1991). One of the most recent has focused on the role of calcium in chemically induced toxicity and is based on a scheme put forward by Orrenius and coworkers (Orrenius, 1985; Orrenius and Nicotera, 1987; Orrenius *et al.*, 1988). They proposed that toxicants that bind thiol compounds not only deplete glutathione, but also inactivate a number of proteins, ie. Ca^{2+} ATPases, that are responsible for calcium homeostasis in the cell. This combined insult causes a sustained intracellular calcium elevation with resultant activation of endonucleases, phospholipases and proteases that lead to cell death.

Several different laboratories utilizing a variety of cell culture systems have examined the effect of HD ($\sim$ 100 µM - 1.0 mM) on calcium and have come to varying conclusions as to the importance of calcium homeostasis in HD-induced cytotoxicity. The first laboratory to test the thiol-Ca^{2+} hypothesis found that in mouse fibroblast B77 cells, HD induced a modest, but immediate and sustained rise in intracellular calcium levels that was independent of external calcium concentrations (Hua *et al.*, 1993). However, follow-up work by Mol and coworkers (1994) were equivocal. In second passage cultures of human skin epidermal keratinocytes grown on 3T3 feeder-layers, they found little or no evidence of HD-induced intracellular calcium elevation and dismissed the small increases they detected as reflecting abnormal cellular physiology rather than acute toxicity (Mol, 1994; Mol and Smith, 1996). In contrast, intracellular calcium was consistently found to be elevated 2- 6 h after HD exposure in a mouse neuroblastoma-rat glioma hybid cell line (NG108-15) by Ray and colleagues (Ray *et al.*, 1995), who also found a similar elevation in human epidermal keratinocytes (Ray *et al.*, 1994). This

same group has gone on to report that the cell-permeant calcium chelator BAPTA-AM not only prevented this intracellular calcium elevation, but was also effective in preventing HD-induced cytotoxicity (Ray *et al.*, 1996; 1997; 1998). Recent studies in our laboratory have tended to confirm the initial findings of Hua *et al.*, 1993. In first passage just-confluent cultures of neonatal human skin keratinocytes, we found that HD does indeed cause an immediate, concentration-dependent and sustained elevation of intracellular calcium that appeared to originate from intracellular stores. Furthermore, the sensitivity to HD and the time course of this calcium elevation roughly paralleled the effects of HD on cell viability, although the concentrations of HD that maximally elevated intracellular calcium would be considered supralethal (Hamilton *et al.*, 1998). In order to further assess the link between intracellular calcium and HD-induced cytotoxicity we examined the effect of modulating both external and internal calcium levels prior to HD culture exposure, on the resultant toxicity.

Human keratinocyte culture is an extremely dynamic system whose sensitivity to HD is dependent on a large number of variables, not the least of which is the confluency of the cultures. These variables may well be responsible for the different findings concerning the effects of HD on intracellular calcium. The differentiative state of both mouse and human keratinocyte culture has been extensively characterized as being dependent on the intracellular calcium environment, which is dependent on a number of factors, including extracellular calcium levels and the confluency of the culture (Hennings *et al.*, 1980; Kruszewski *et al.*, 1991; Pillai *et al.*, 1990; Sharpe *et al.*, 1989; Yuspa *et al.*, 1989). For these reasons we examined the effects of a variety of treatments in both actively proliferating and just-confluent cultures. We found that HD was several times more toxic in actively proliferating, subconfluent cultures than it was in confluent

cultures where a large proportion of the cells would be committed to terminal differentiation. We have not been successful using proliferating keratinocytes in our calcium studies because proliferating cells load poorly or not at all with Fura-2. However, the sensivity of confluent cells to HD toxicity, although reduced, was similar to the concentrations required to elevate intracellular calcium levels (Hamilton *et al.*, 1998).

We attempted to replicate the work of Ray and coworkers with BAPTA-AM (Ray *et al.*, 1996; 1997; 1998). This cell-membrane permeable calcium chelator should modulate toxicity if intracellular calcium elevation is indeed causal or involved in the cascade of events that lead to HD toxicity. Preliminary studies parallelled this group's work and found that at 25 μM, BAPTA-AM was non-toxic in confluent cultures. In contrast, proliferating cells were very sensitive to this compound, with an LC_{50} of 20.7 μM +/- 8.9 μM. Unexpectedly, however, pretreatment of either proliferating or confluent cultures with this chelator failed to modulate HD toxicity in any way, even at BAPTA-AM concentrations that were in themselves toxic. It is unclear why our results contrast those of Ray *et al.* so dramatically. We also carried out experiments with thapsigargin, an endoplasmic reticulum Ca^{2+} ATPase inhibitor, that we have previously shown to eliminate the intracellular calcium elevation induced by HD (Hamilton *et al.*, 1998). The results were similar to those obtained with BAPTA-AM; proliferating cells were very sensitive to this compound ($LC_{50} = 0.8$ μM +/- 0.1 μM), while confluent cultures were not affected at the concentrations used. Furthermore, thapsigargin pretreatment failed to modulate HD toxicity in any way at any concentration used. It appears that, at least in first passage neonatal human skin keratinocytes, modulation of intracellular calcium is not causal in HD toxicity.

We next examined the effects of modulating the calcium concentration of the medium

13

bathing the cultures, since internal calcium levels are known to be sensitive to external calcium. Confluent cultures were totally resistent to variations in external calcium, even at nominal zero calcium concentrations. However, proliferating cultures were extremely sensitive to changes in external calcium and rapidly lost viability as the calcium concentration decreased below 75 μM. Although the concentration-response of HD in confluent cultures did not vary with external calcium concentrations from 0 - 100 μM, we examined the effect of varying calcium concentration in conjuction with optimal concentrations of BAPTA-AM (25 μM) and thapsigargin (10 μM). Although unlikely, it was possible that when these compounds exhaust internal stores, external calcium plays a role in HD toxicity at later time points than those measured with Fura-2. This possibility was ruled out when once again, no treatment or combination of treatments had any effect on HD toxicity.

The last series of experiments examined the effects of the calcium ionophore ionomycin. Ionomycin concentrations higher than 1.0 μM were toxic in both proliferating and confluent cultures with the former being the most sensitive. Pretreatment of cultures with this compound at low (100 μM) and high (1.2 mM) calcium concentrations were carried out only in confluent cultures. The "calcium switch", from low to high calcium, is well known to induce terminal differentiation in proliferating keratinocytes and interpretation of the effects of HD in this situation would be difficult. As with every other experimental regimen designed to perturb calcium homeostasis in these studies, ionomycin had no effect on the toxicity of HD, even at 1.2 mM calcium.

In summary, earlier work in this laboratory has shown that HD induces a rapid, sustained, concentration-dependent increase in intracellular calcium in neonatal human skin keratinocytes

that appears to originate from intracellular stores. However, this study shows that perturbation of calcium homeostasis is not causal or directly involved in the development of HD-induced toxicity, at least in this cell culture system.

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LEGENDS

Figure 1 Toxicity of HD in proliferating and confluent cultures of human skin keratinocytes. Proliferating (3 days, X) and confluent (7 days, O) cultures of neonatal human skin keratinocytes were treated with varying HD concentrations and viability was determined 48 h later using alamarBlue™. Results represent a typical experiment utilizing 6 wells per data point (mean +/- SD).

Figure 2 Effects of thapsigargin and BAPTA-AM in human skin keratinocytes. The toxicity of BAPTA-AM (panel a) and thapsigargin (panel b) was examined in both proliferating (X) or confluent (O) cultures. Both compounds were markedly more toxic in proliferating cultures than in confluent cells. The modulatory effect of BAPTA-AM (panel c) and thapsigargin (panel d) on the toxicity of HD in proliferating (open bars) and confluent (crosshatched bars) keratinocyte cultures was investigated by incubating test drugs with the cultures for 1 h prior to HD exposure and assaying for viability 48 hours later. Results represent the mean +/- SD of 3 experiments and are normalized against the LC_{50} of vehicle-pretreated, HD-exposed cultures (LC_{50} of drug-pretreated, HD-treated cultures divided by the LC_{50} of vehicle-pretreated, HD-treated cultures x 100). Neither treatment had any effect on HD toxicity.

Figure 3 Effect of extracellular calcium concentration on the viability of proliferating and confluent keratinocytes. Cultures were grown in KSFM until the day of treatment on day 3 (proliferating, X) or day 7 (confluent, O) of culture. At this time the cultures were aspirated, rinsed twice with 200 μL of buffer and then refed with calcium-free medium that had been

adjusted to the desired calcium concentration with calcium chloride. The cultures were then assayed for viability 48 h later. Results represent the mean +/- SD of 3 experiments and are normalized against the LC_{50} of cultures incubated at 100 μM calcium. Reduced external calcium levels were toxic in proliferating cells, but had no effect in confluent cultures.

Figure 4 Effect of BAPTA-AM and thapsigargin on HD toxicity in human keratinocyte culture at different extracellular calcium concentrations. Confluent keratinocyte cultures grown in normal culture medium were changed to medium adjusted to 0.0 to 100 μM calcium. After 1 h the cultures were incubated with BAPTA-AM (25 μM) or thapsigargin (5.0 μM) for an additional 1 h prior to HD exposure. The figure shows the results of HD treatment only (cross-hatched bars), BAPTA-AM pretreatment (open bars) or thapsigargin pretreatment (closed bars). Results represent the mean +/- SD of 3 experiments and are normalized against the LC_{50} of vehicle-pretreated, HD-exposed cultures incubated in medium containing 100 μM calcium (the LC_{50} of drug-pretreated, HD-treated cultures divided by the LC_{50} of vehicle-pretreated, HD-treated cultures x 100). No treatment yielded results that were different than another.

Figure 5 Effect of ionomycin in keratinocyte culture at low or high calcium concentration. The toxicity of ionomycin was examined in confluent (X) or proliferating (O) cultures by treating the cells with varying concentrations of test compound and assaying for cell viability 48 h later (Fig. 5a). The effect of ionomycin on the toxicity of HD was examined only in confluent human keratinocyte cultures. The cells were grown in normal culture medium and then changed into low calcium (100 μM, open bars) or high calcium (1.2 mM, cross hatched bars) medium. After 1 h

the cultures were incubated with ionomycin for an additional 1 h prior to HD treatment (Fig. 5b). Results represent the mean +/- SD of 3 experiments and are normalized against the LC_{50} of vehicle-pretreated, HD-exposed cultures (the LC_{50} of drug-pretreated, HD-treated cultures divided by the LC_{50} of vehicle-pretreated, HD-treated cultures x 100). Ionomycin had no effect on the toxicity of HD.

Figure 6 Effect of HD or drug treatment on intracellular free calcium levels in human keratinocytes. 1.0 mM HD induced an immediate and sustained rise in intracellular levels as reflected by the rise in fluorescence ratio values (panel a). The fluroescence ratio value is defined as the signal intensity at 340 nm (maximum for Ca^{2+} associated FURA 2) divided by the signal at 380 nm (minimum for Ca^{2+} associated FURA 2). Thapsigargin caused an immediate rise in intracellular calcium that returned to baseline values. In contrast, ionomycin induced a sustained rise in intracellular calcium that was immediately eliminated when the calcium chelator BAPTA was introduced into the cuvette (panel b).

Figure 1
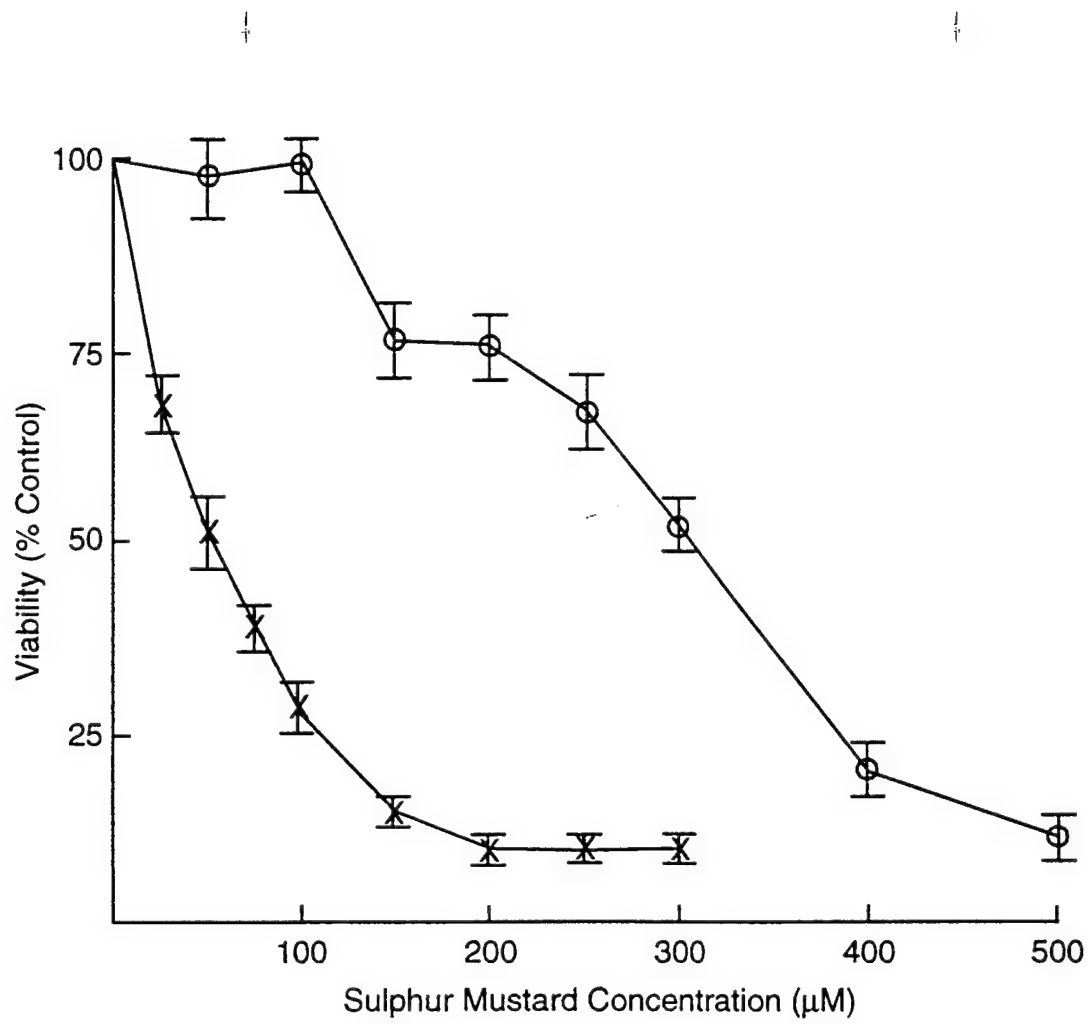
Figure 2
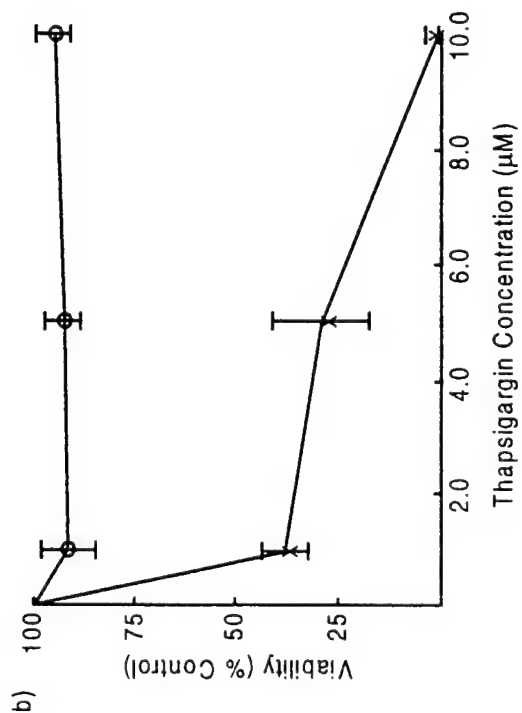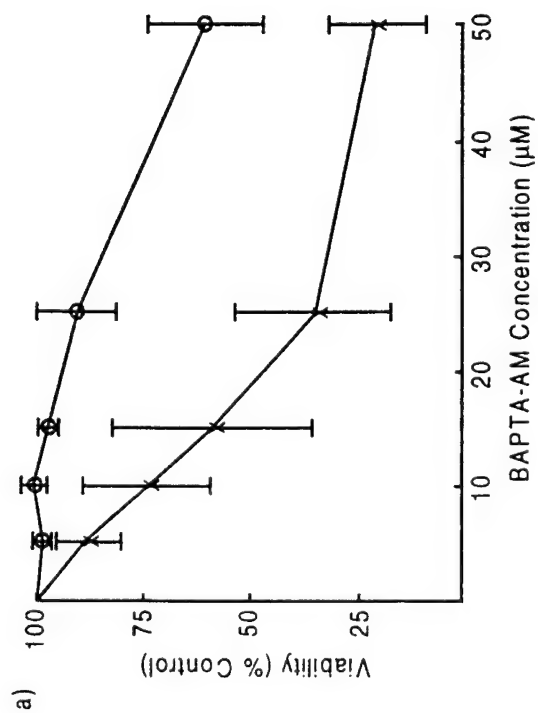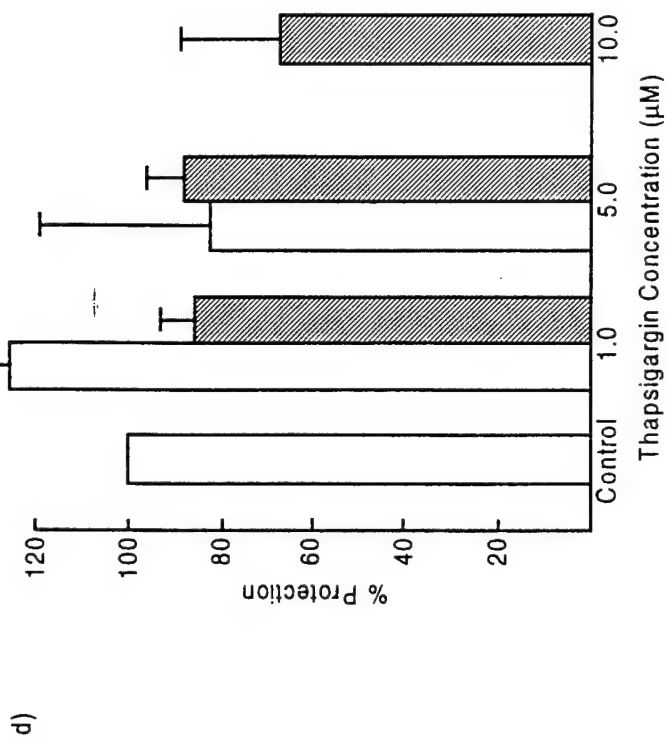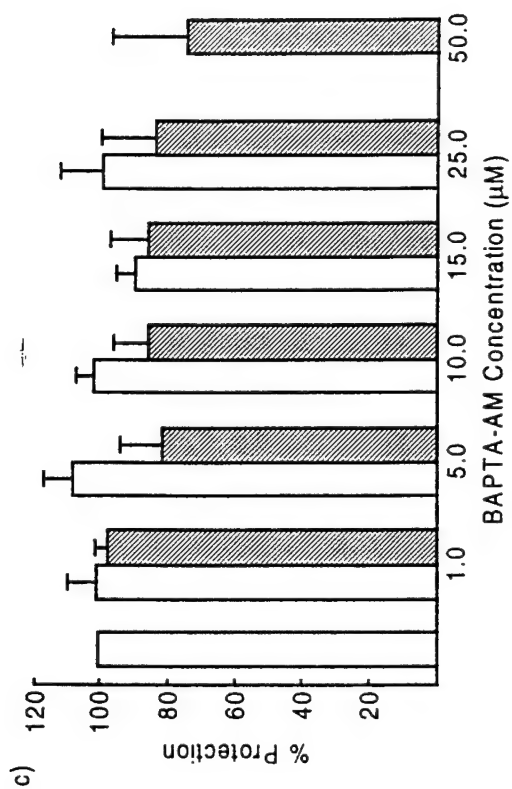
Figure 3
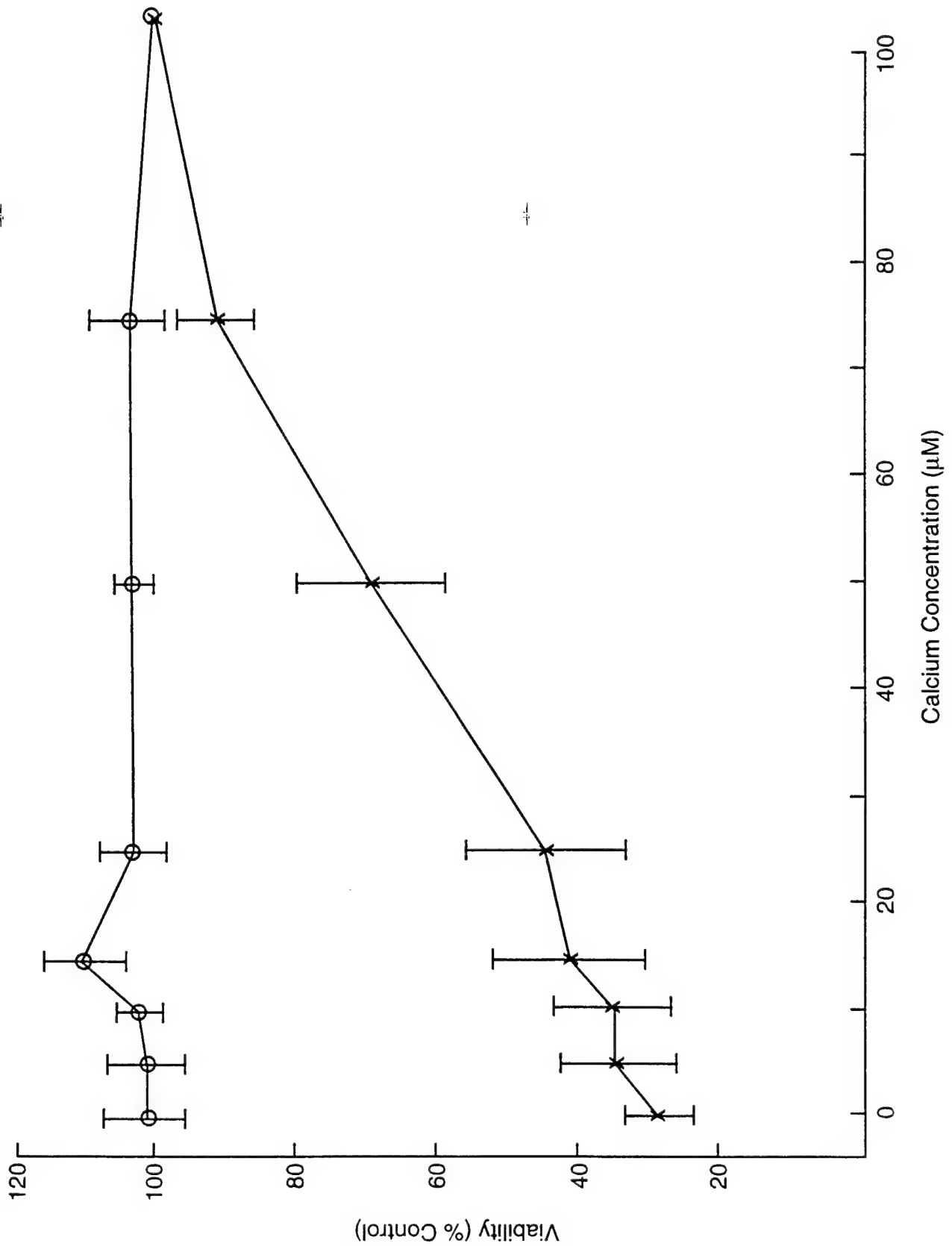
Figure 4

Figure 5

Figure 6

a)

b)
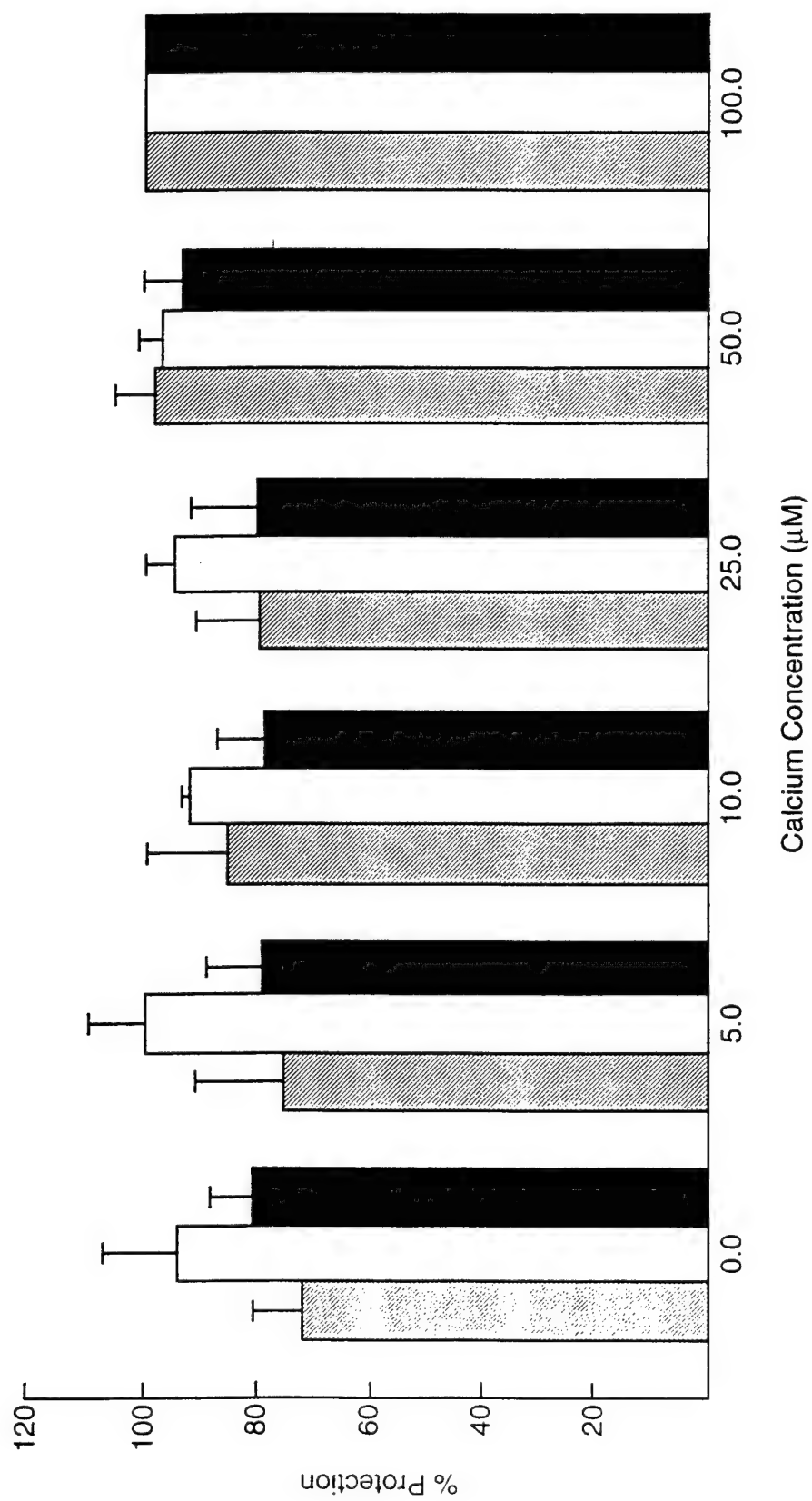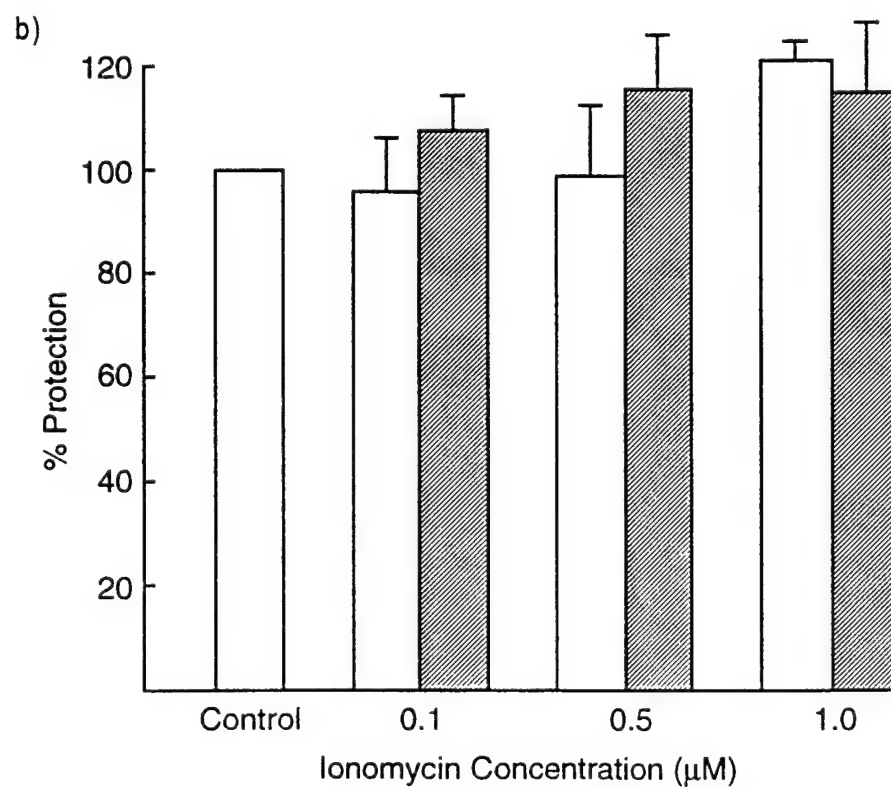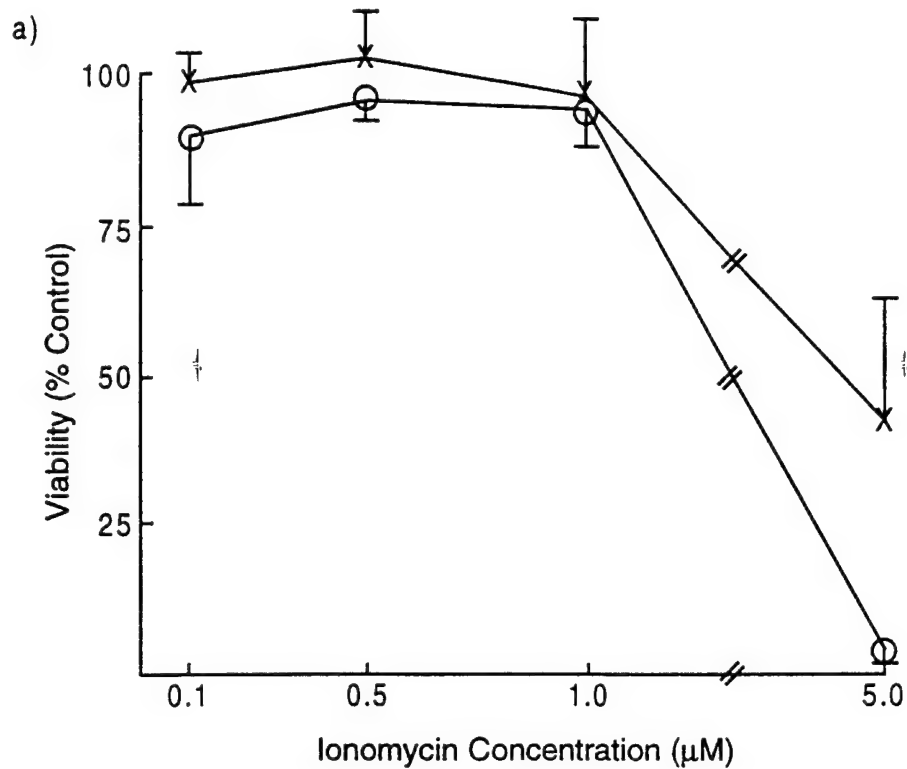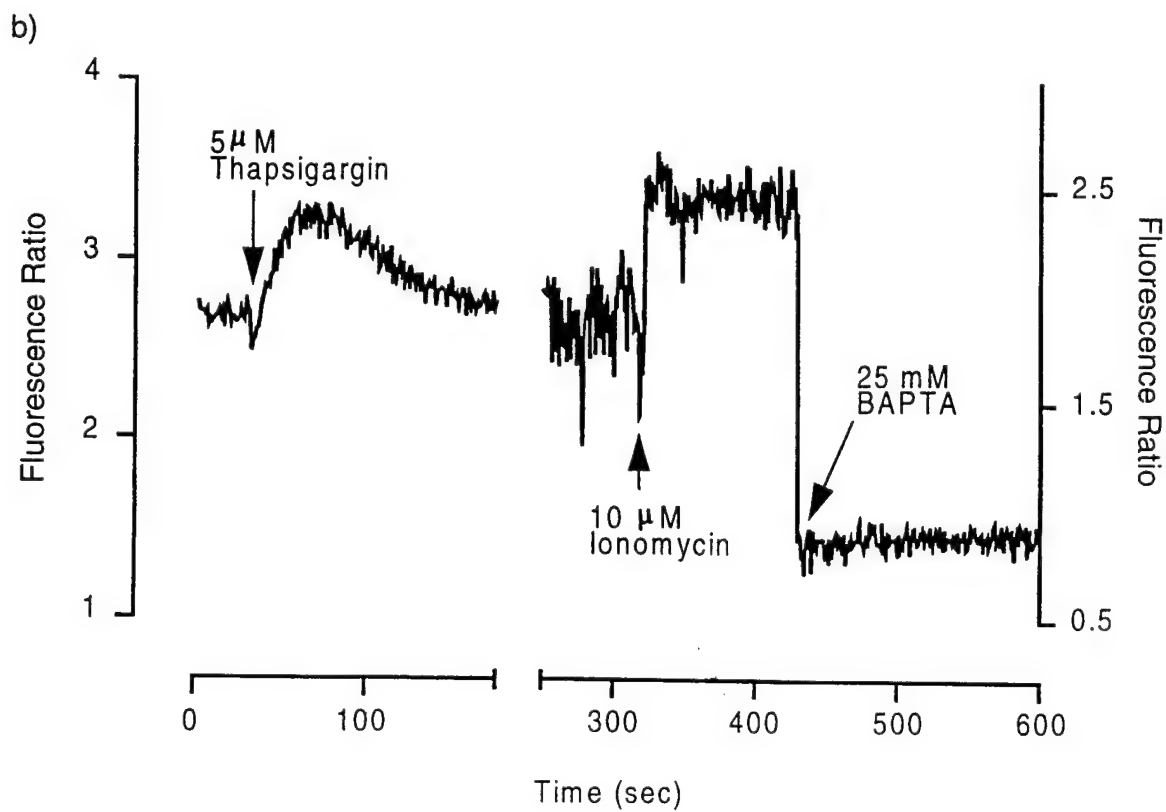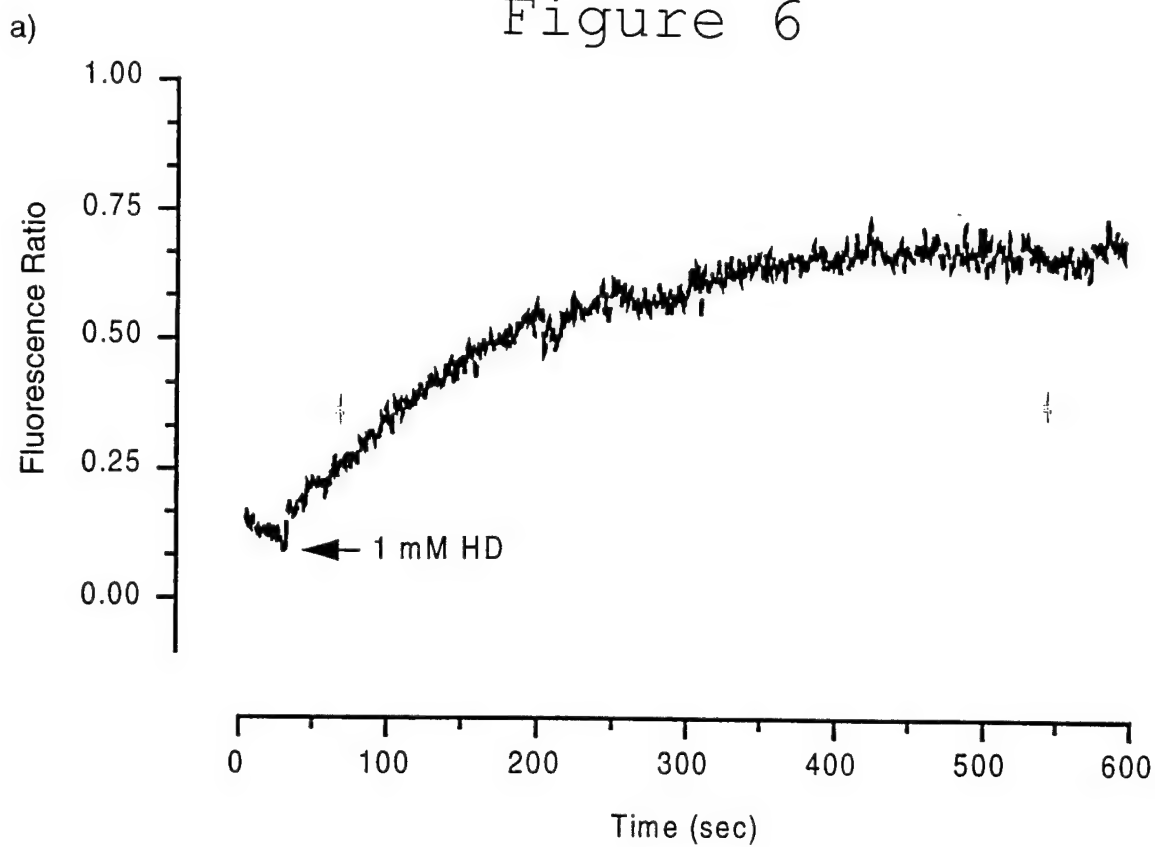
ANNEX B

Figures 1 to 23
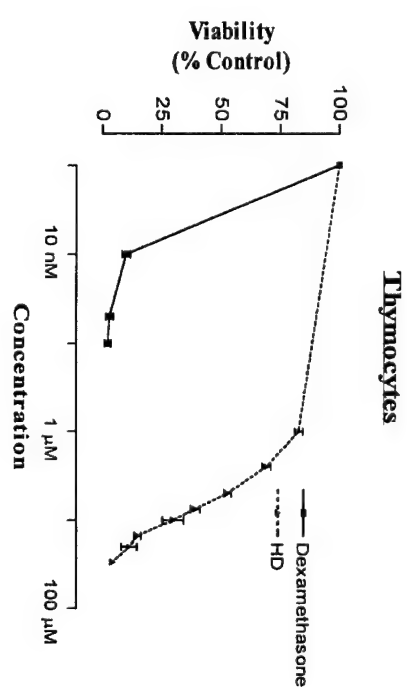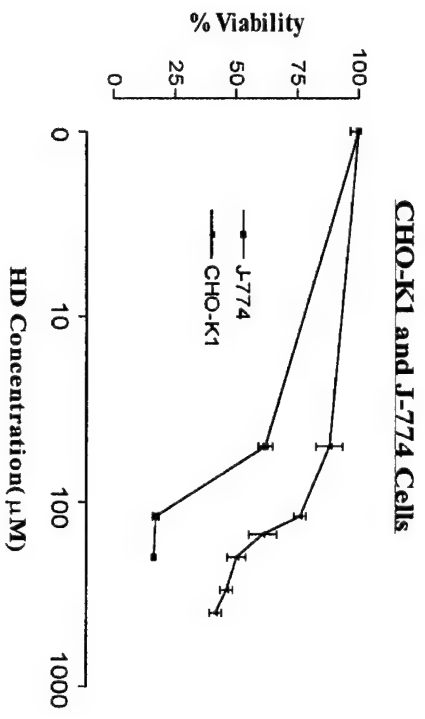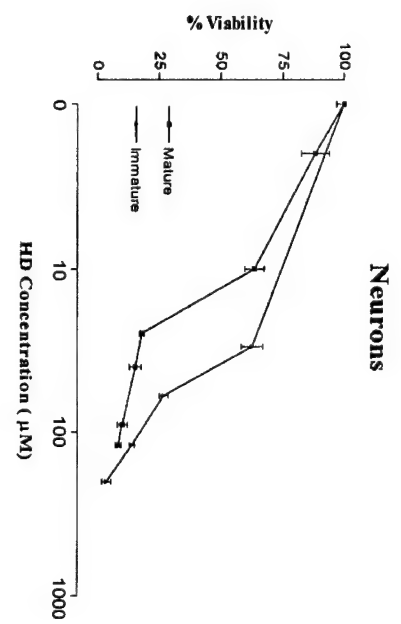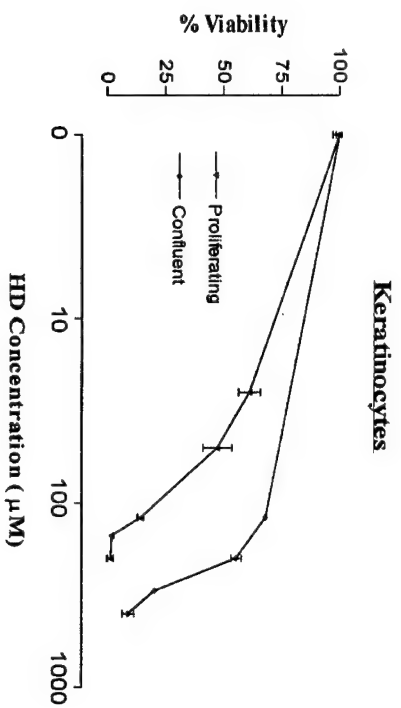
Figure 1

Cytotoxicity of HD in a Variety of Cell Types

Dose-response to HD in 5 cell types. Dexamethasone
is included as a positive control for inducing apoptosis in thymocytes.
See Table 1 for LC$_{50}$s

0 μM 50 μM 100 μM 200 μM
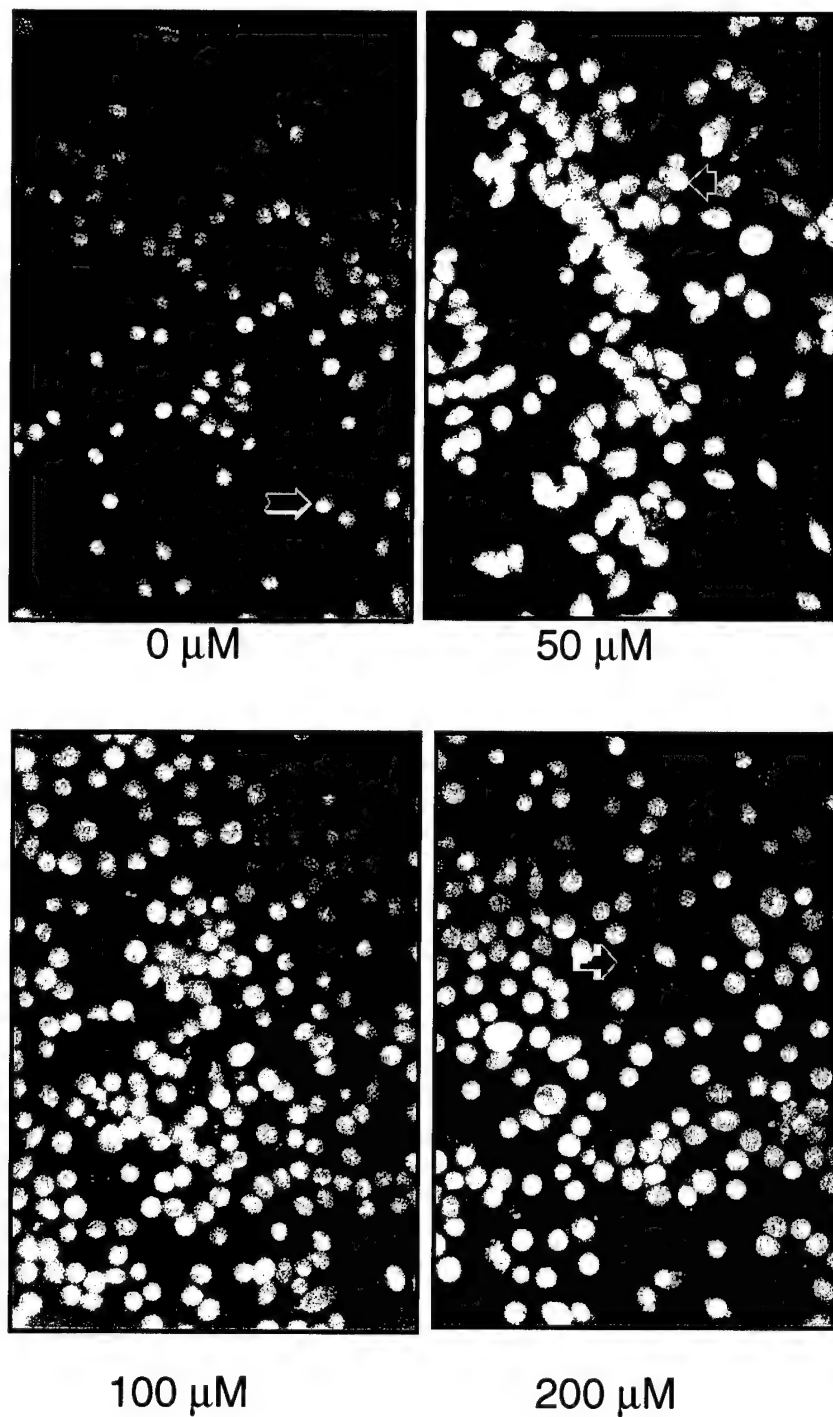
Figure 2

Acridine Orange/Ethidium Bromide Staining of J774 Cells Treated by
Different Concentrations of HD for 4 Hours
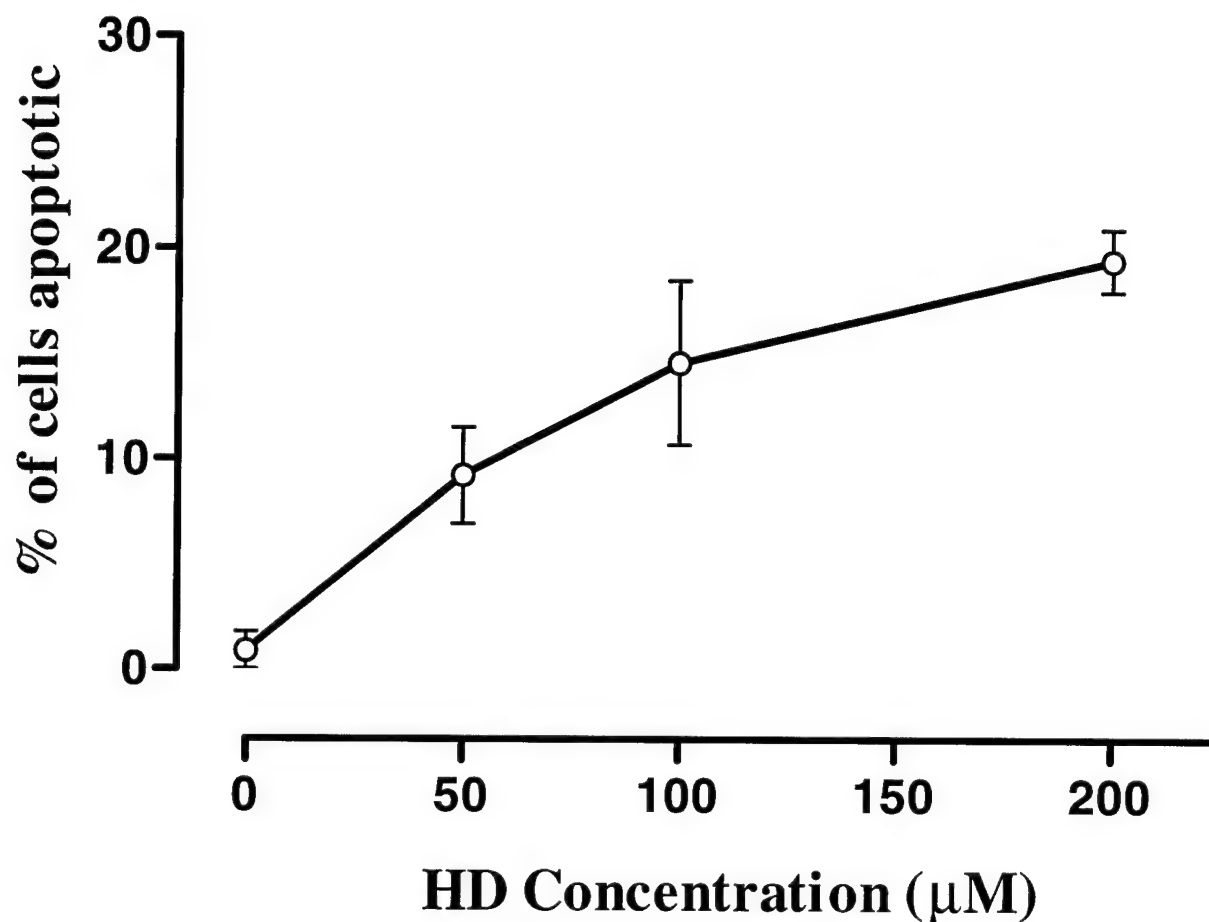
Figure 3

HD Induced Apoptosis of J774 Cells by TUNEL assay

Cells were exposed to HD for 6 hours and then assayed by the TUNEL assay as described in Methods. Apoptosis increased in response to increasing HD concentrations up to about 200 μM. Results are means ± SEM from 3 independent experiments in triplicate.
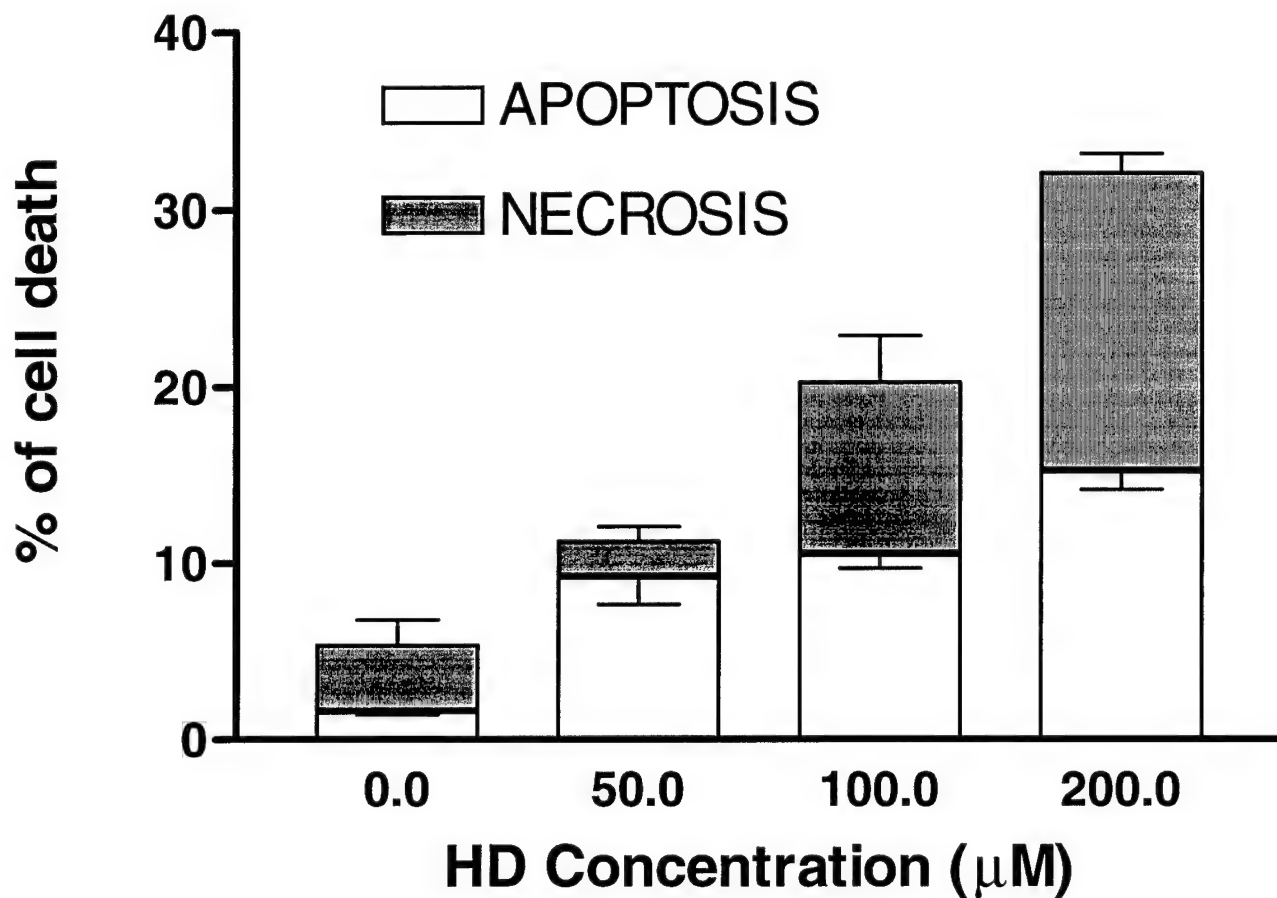
Figure 4

Acridine Orange and Ethidium Bromide Assay of J-774 Cells

Cell death after HD exposure as measured by Acridine Orange and Ethidium Bromide double staining. Note that the percentage of cells dying from necrosis *vs* apoptosis increases at the higher concentrations of HD.

A. Control: CHO-K1 cells were stained uniformly green.

B and C: Cells treated with 400 μM HD and 600 μM HD respectively. The apoptotic cells (indicated with ← as example) show yellowish "dots" of condensed chromatin. The necrotic cells or the apoptotic cells which have lost their membrane integrity appear orange due to co-staining with ethidium bromide (indicated with ⌐ as example).
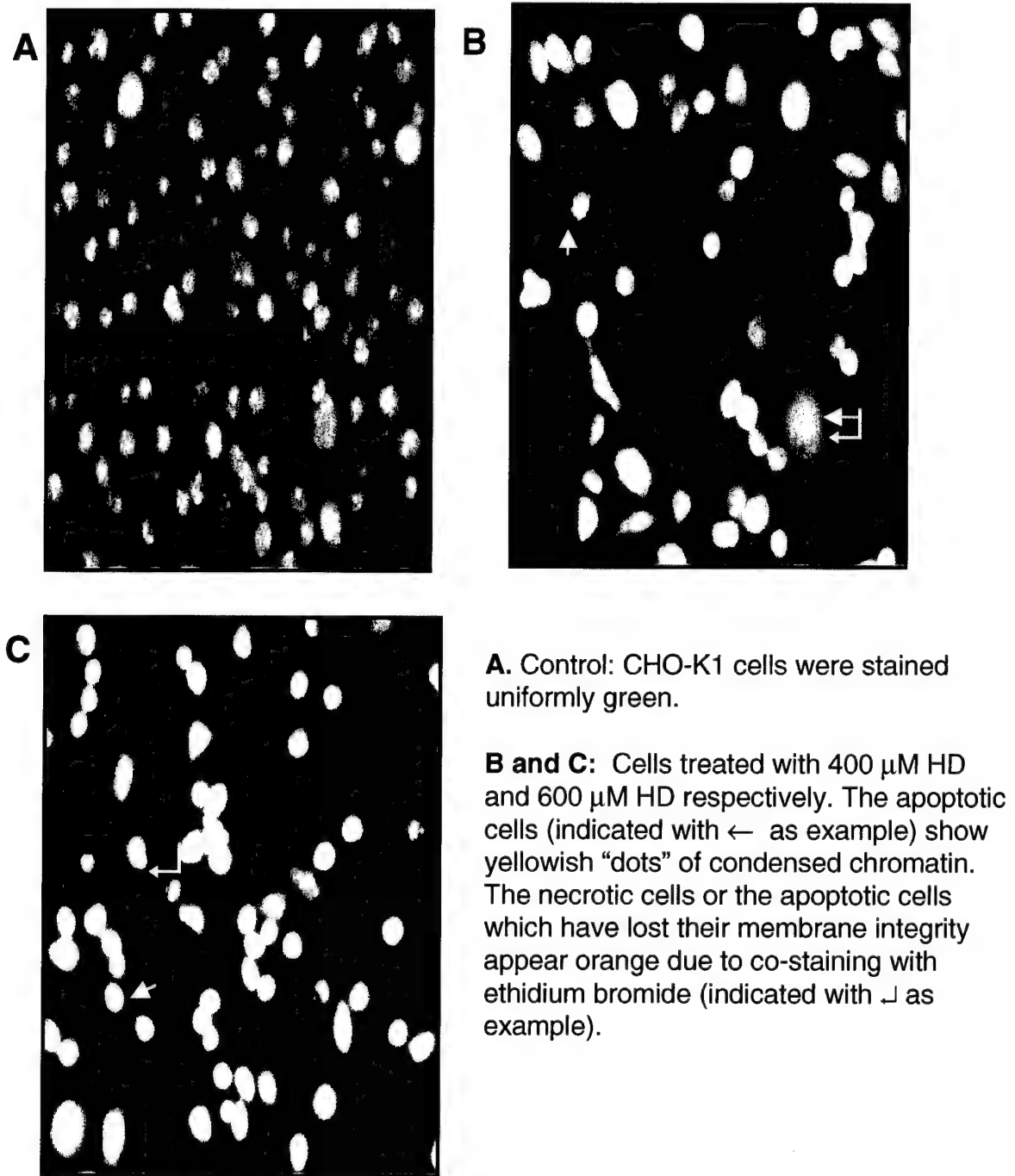
Figure 5

Acridine Orange/Ethidium Bromide Staining of Sulphur Mustard (HD) Treated CHO-K1 Cells

CHO-K1 cells were treated for 6 hours with or without different concentrations of HD. They were stained with acridine orange/ethidium bromide as described before.
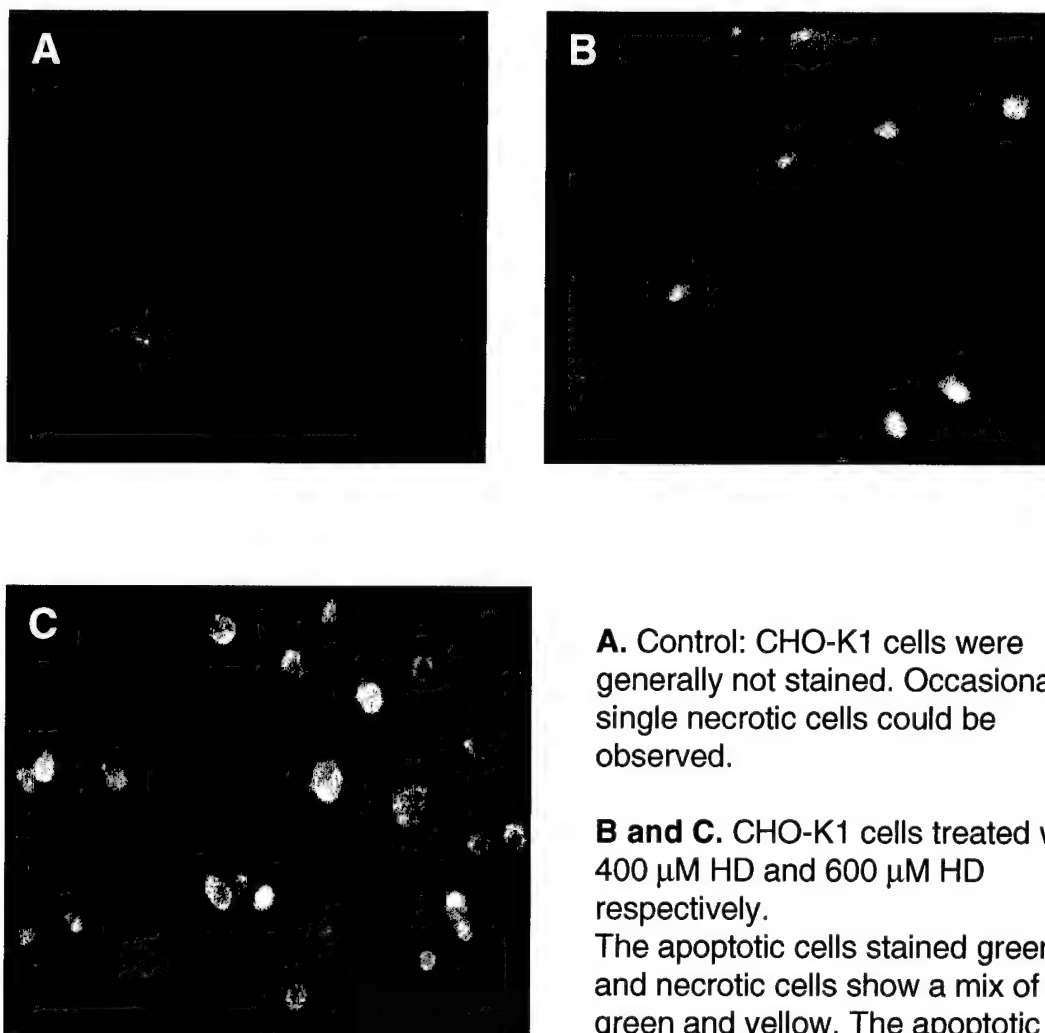
A. Control: CHO-K1 cells were generally not stained. Occasionally, single necrotic cells could be observed.

B and C. CHO-K1 cells treated with 400 μM HD and 600 μM HD respectively.
The apoptotic cells stained green and necrotic cells show a mix of green and yellow. The apoptotic cells are relatively small compared with necrotic cells.

Figure 6

Annexin V and Propidium Iodide Staining of Sulphur Mustard (HD)
Treated CHO-K1 Cells

CHO-K1 cells were treated for 6 hours with or without different concentrations of HD and then stained with Annexin V and propidium iodide as described before.

A. Control

B. HD 200 μM
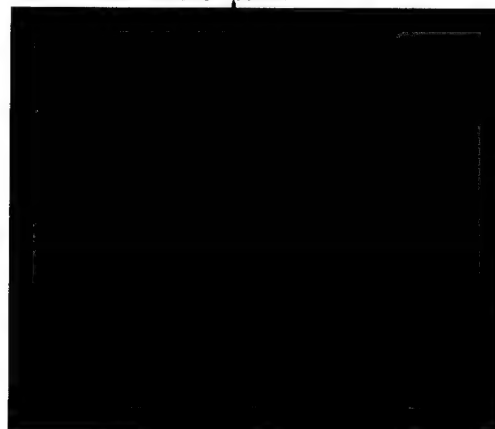
C. HD 400 μM
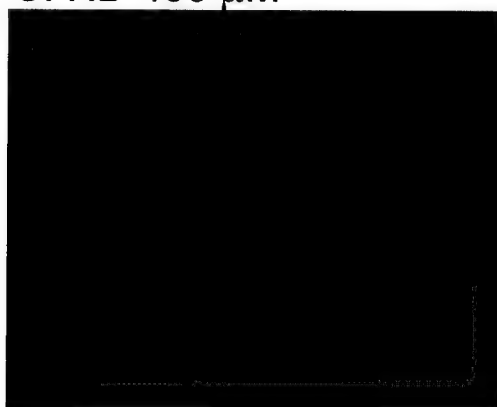
Figure 7

TUNEL Reaction Following CHO-K1 Cells Treatment with Sulphur Mustard (HD)

Typical staining of CHO-K1 cells following 6 hour exposure to HD.
Results of these experiments are quantitated in Table 2.

Figure 8

Sulphur Mustard (HD) Induced DNA Fragmentation in CHO-K1 Cells

DNA fragments were extracted and separated on 1 % agarose gel.
This figure shows representative results of DNA fragmentation following 6 hours of
exposure to HD at two concentrations.

A. Untreated

B. HD 25 µM

C. HD 60 µM
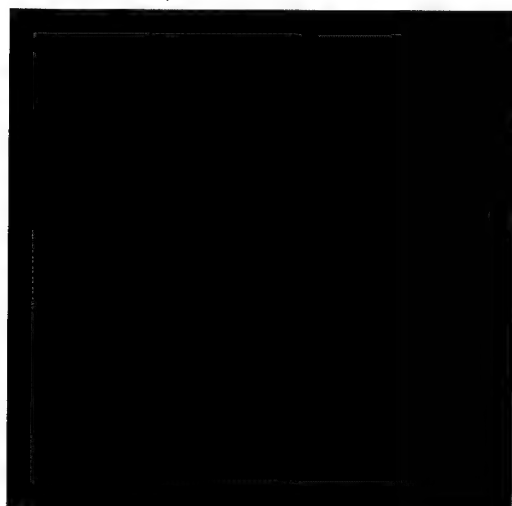
Figure 9

Detection of Apoptotic Chick Neuron Cells Treated
with Sulphur Mustard (HD).

Chick neurons were incubated with HD for 6 hours and assayed for
apoptosis using the TUNEL reaction. Apoptotic cells are identified
as green fluorescent objects.
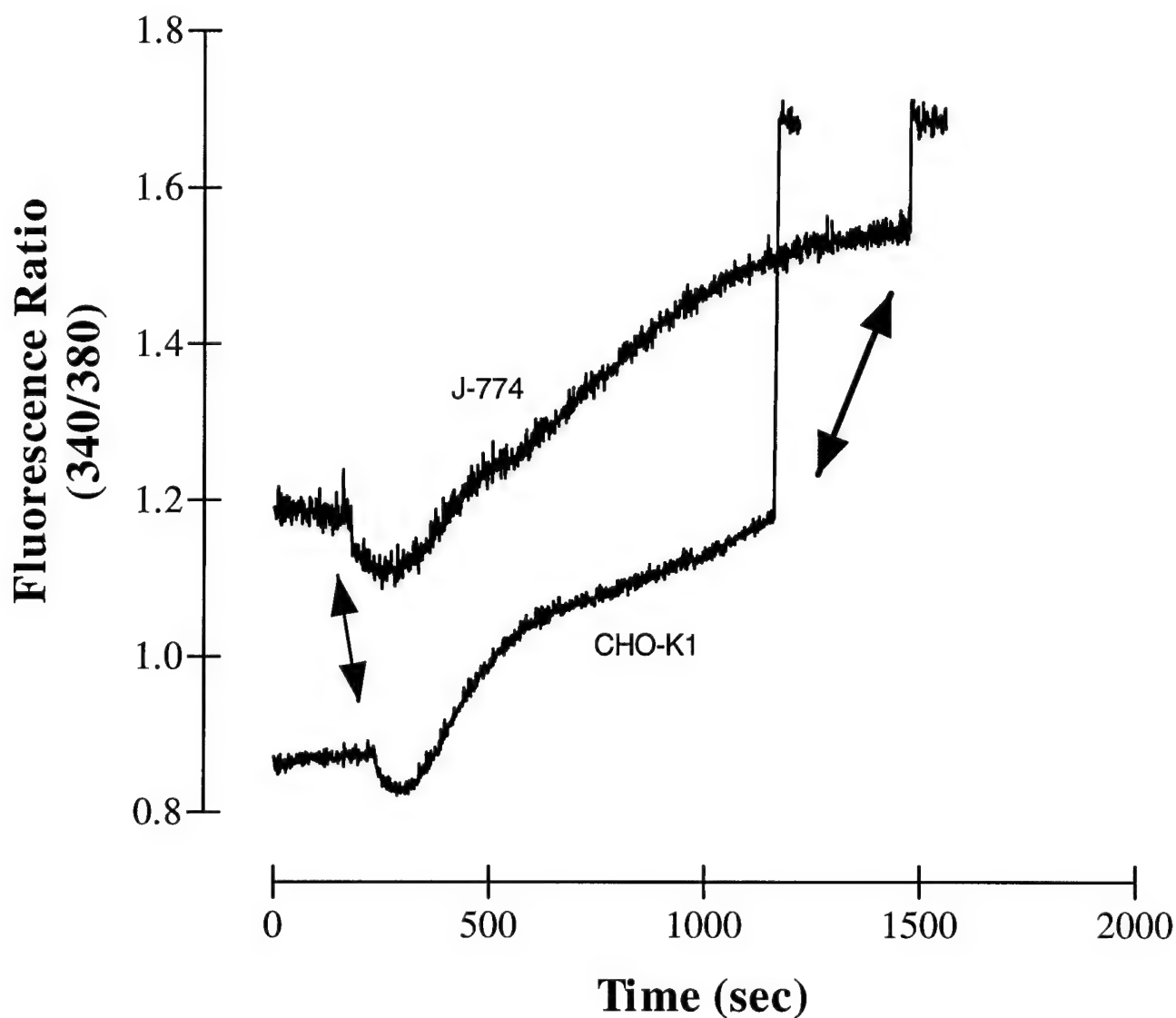
Figure 10

Effect of Sulphur Mustard on $[Ca^{2+}]_{INT}$ in CHO-K1 and J774 Cells

The addition of 600μM HD (first arrow) caused a slow increase in the 340/380 fluorescence ratio (indicative of intracellular Ca^{2+}) in both CHO-K1 and J774 cells. These results are similar to those found in human keratinocytes (Hamilton *et al.*, 1999) and were replicated 3 times with different sub-cultures of these cell lines. (Ionomycin 1μM was added at the second arrow).
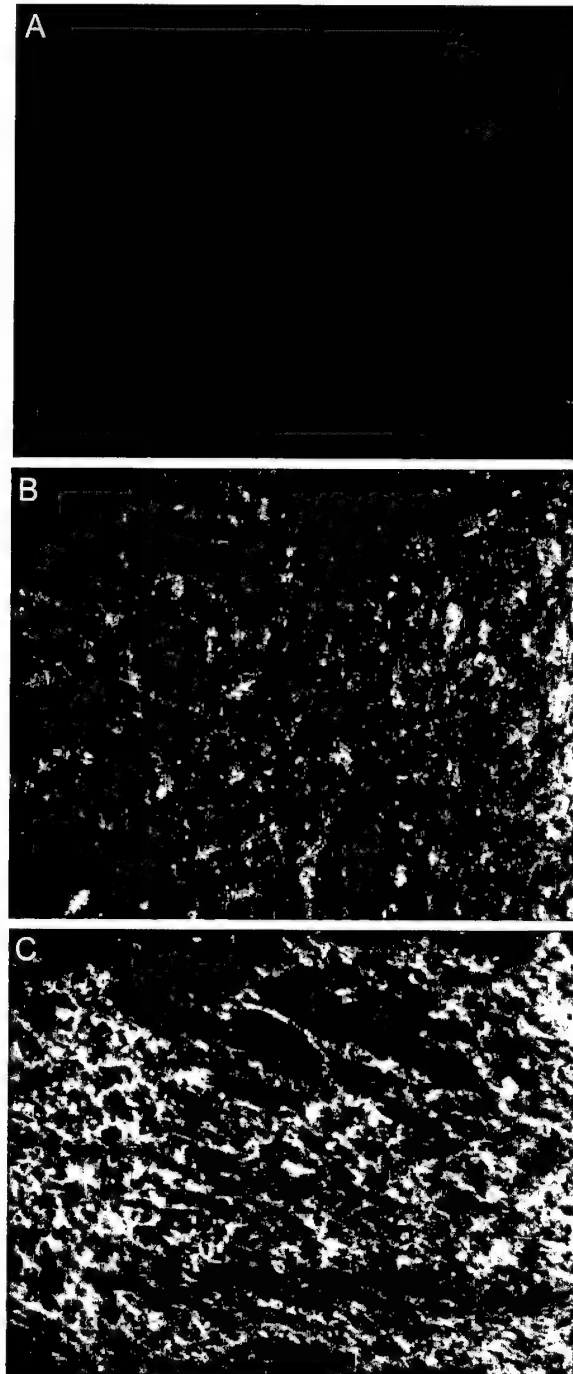
Figure 11

Immunohistochemical Detection of P$_2$X Receptor Expression in Rat Brain

Thin sections of whole rat brain were incubated with anti-P$_2$X antibodies. Tissues stained positively (green fluorescence) for both P$_2$X$_1$ (B) and P$_2$X$_7$ (C) purinoreceptors. Control slides were negative (A).
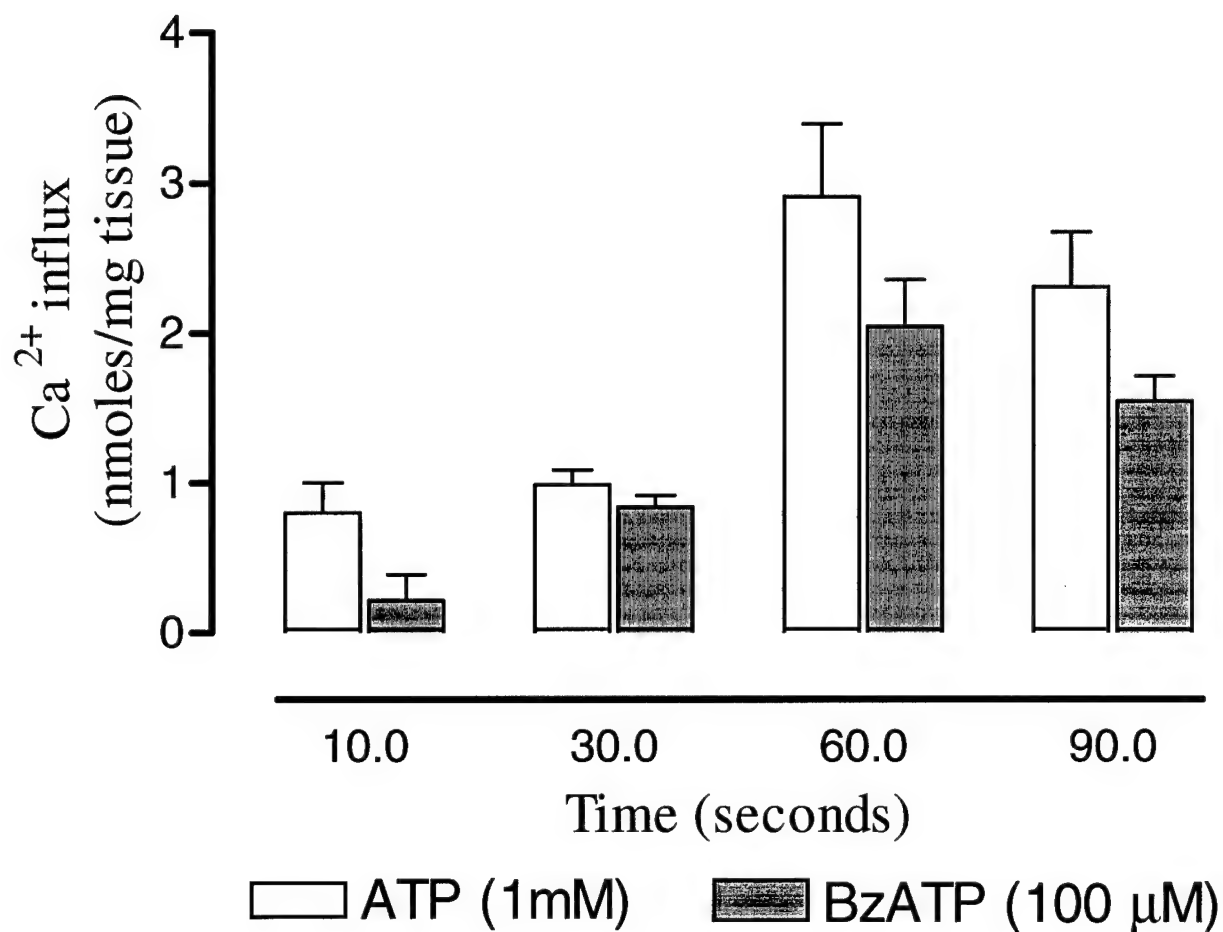
Figure 12

Effect of Incubation Time on Ca^{2+} Influx in Rat Brain Synaptosomes

ATP and benzyl-ATP were incubated with crude rat synaptosomes at the concentrations indicated. Maximal evoked Ca^{2+} influx occurred at 60 sec. Results are means ± SEM from 3 experiments in triplicate.
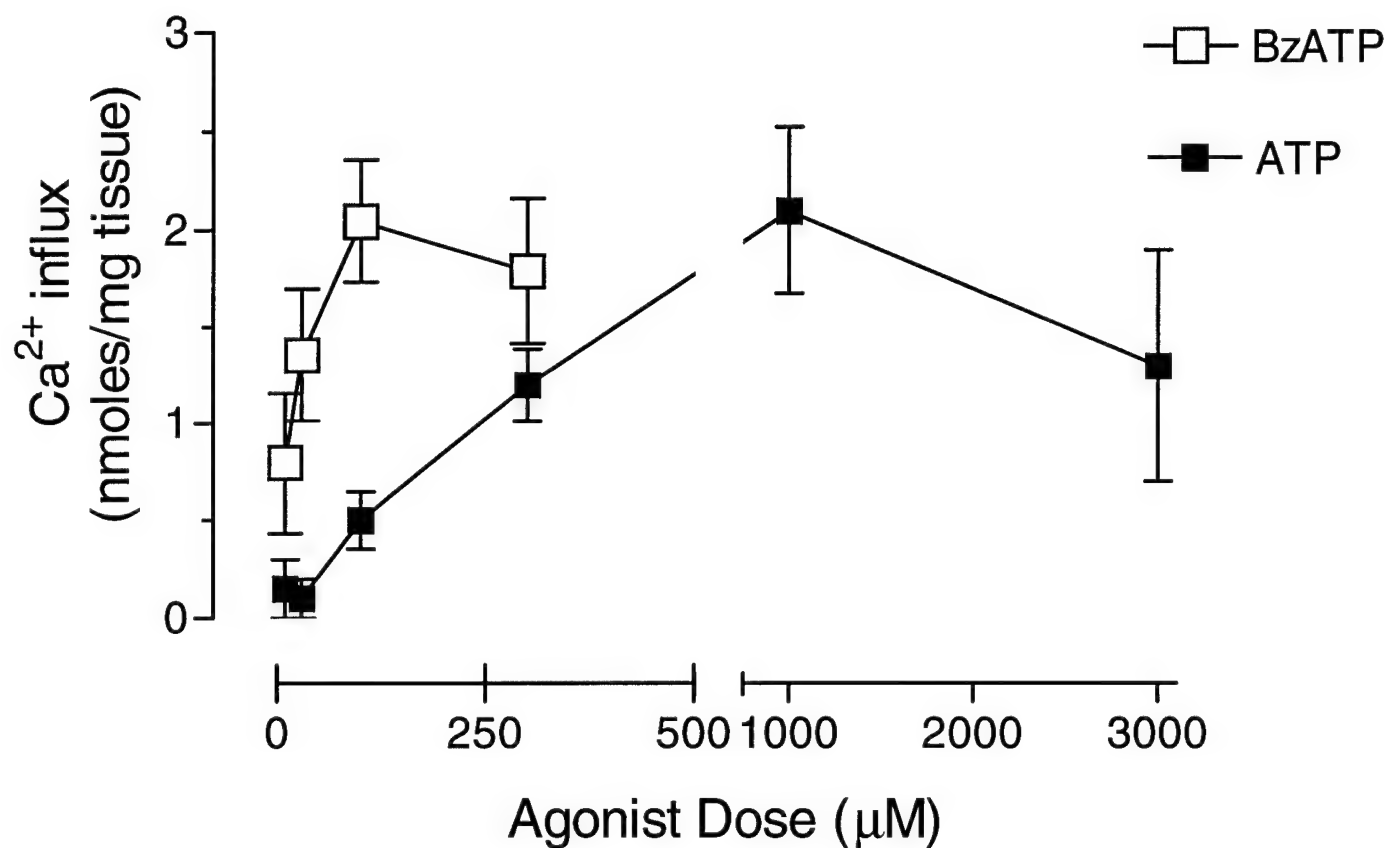
Figure 13

Nucleotide Stimulated Ca^{2+} Influx in Rat Brain Synaptosomes

Synaptosomal accumulation of $^{45}Ca^{2+}$ following
60 sec exposure to ATP or benzyl-ATP. Values are the
average of 3 independent experiments in triplicate ± SEM.
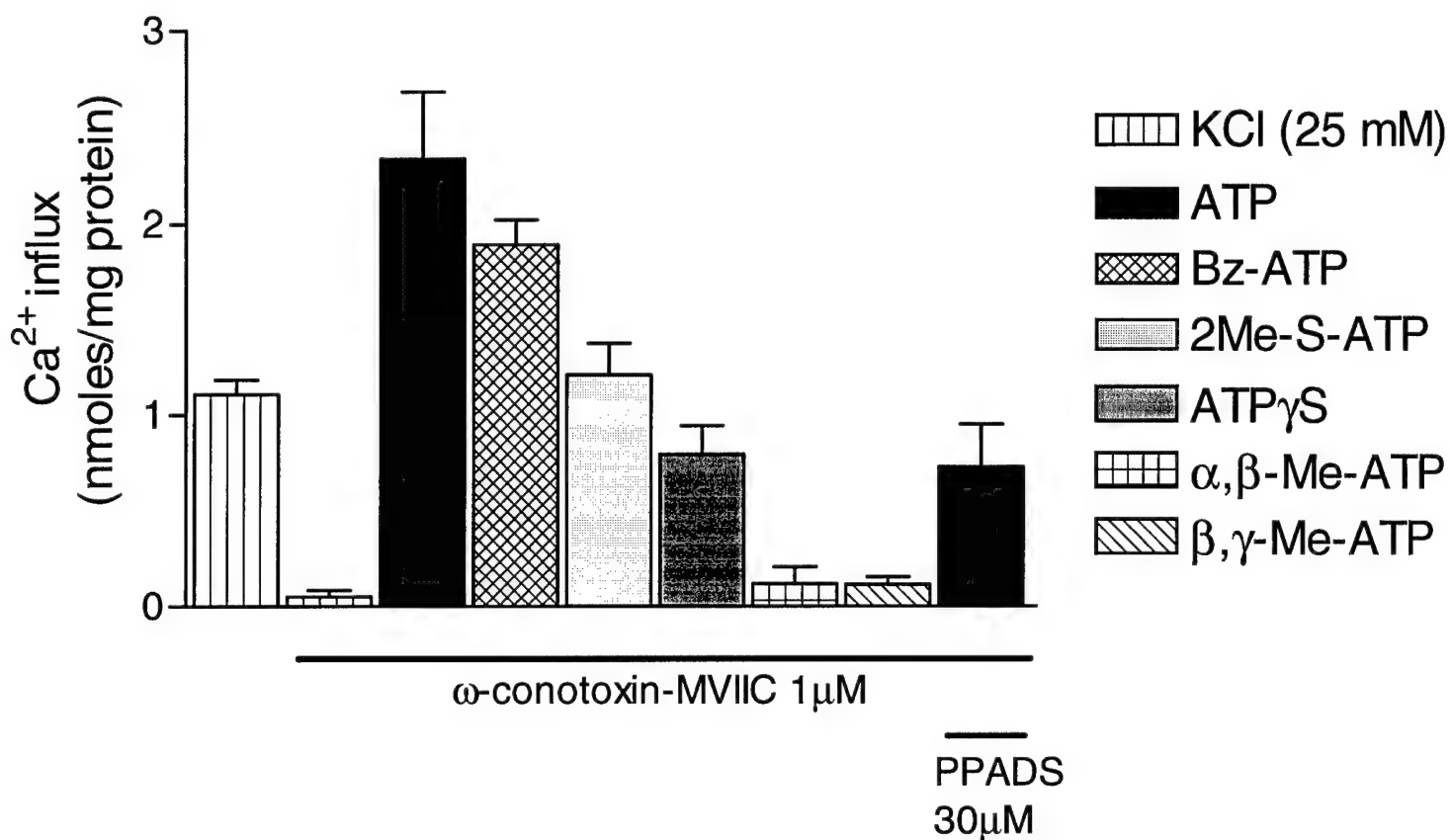
Figure 14

Adenine Nucleotide Evoked Ca^{2+} Influx in Rat Brain Synaptosomes

Structure activity relationship of P_2 receptor agonists on Ca^{2+} influx in rat brain synaptosomes. (All nucleotides were at 1mM). ω-CmTx was added to eliminate voltage sensitive Ca^{2+} channel influx. PPADS is a P_2X receptor antagonist. The SAR suggests the presence of P_2X_7 receptors on these synaptosomes.

A

Vas D   Brain   Thymo   Synap

B

Vas D   Brain   Thymo   Synap
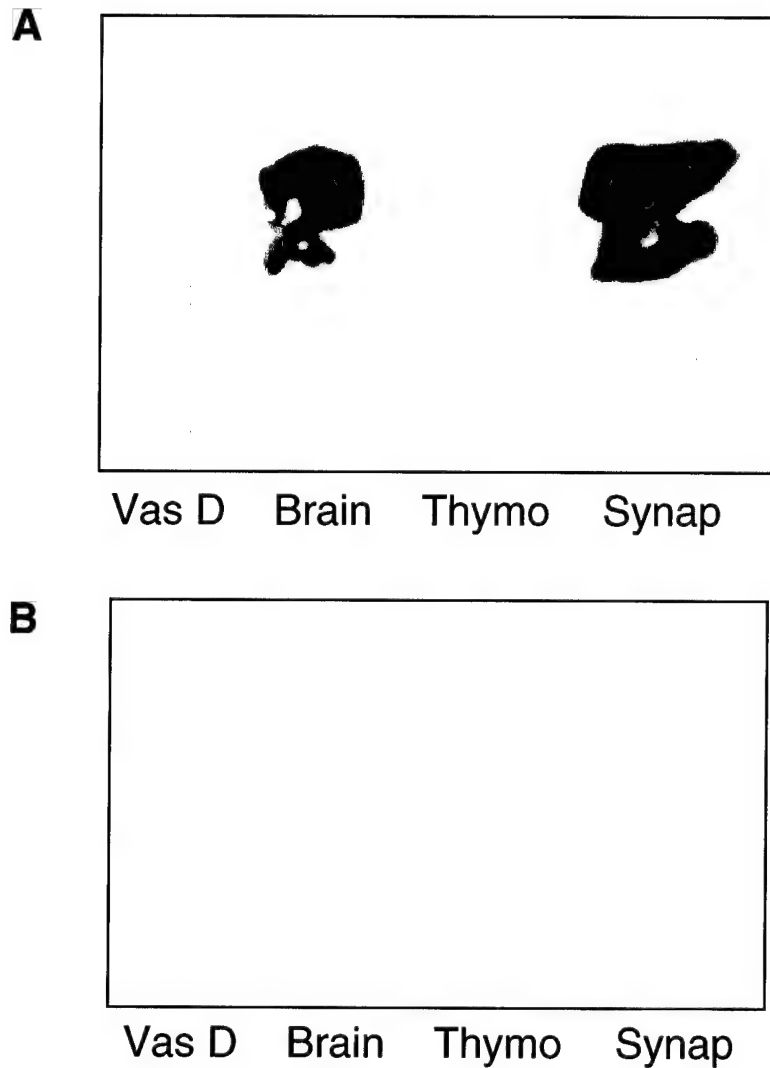
Figure 15

Western-Blotting using Anti-P_2X_7 Antibody from Alomone Labs

Western-Blotting of different tissues using an anti-P_2X_7 antibody from Alomone Labs (corresponding to residues 576-595 of rat P_2X_7). Equal amounts of tissue extracts were loaded on each lane. Vas D: Vas Deferens, Thymo: Thymocyte, Synap: Synaptosome. A specific peptide which blocks the action of this antibody was used in B to confirm its specificity.
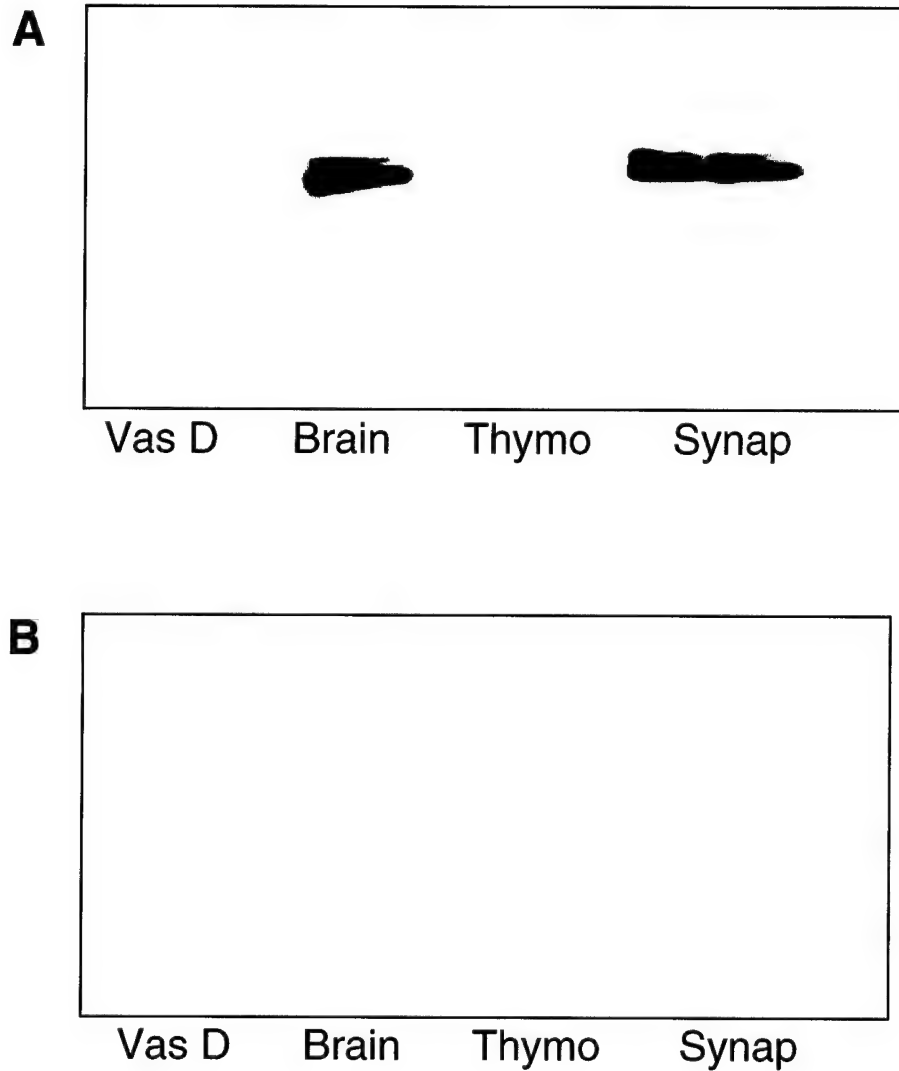
Figure 16

Western-Blotting using Anti-P_2X_7 Antibody from
CHEMICON International, Inc.

Western-Blotting of different tissues using anti-P_2X_7 polyclonal antibody from
CHEMICON International, Inc. (corresponding to residues 457-472 of rat
P_2X_7). Equal amounts of tissue extract were loaded on each lane. Vas D: Vas
Deferens, Thymo: Thymocyte, Synap: Synaptosome. Specific peptide which
blocks the action of this antibody was used in B to confirm the specifity of this
antibody.
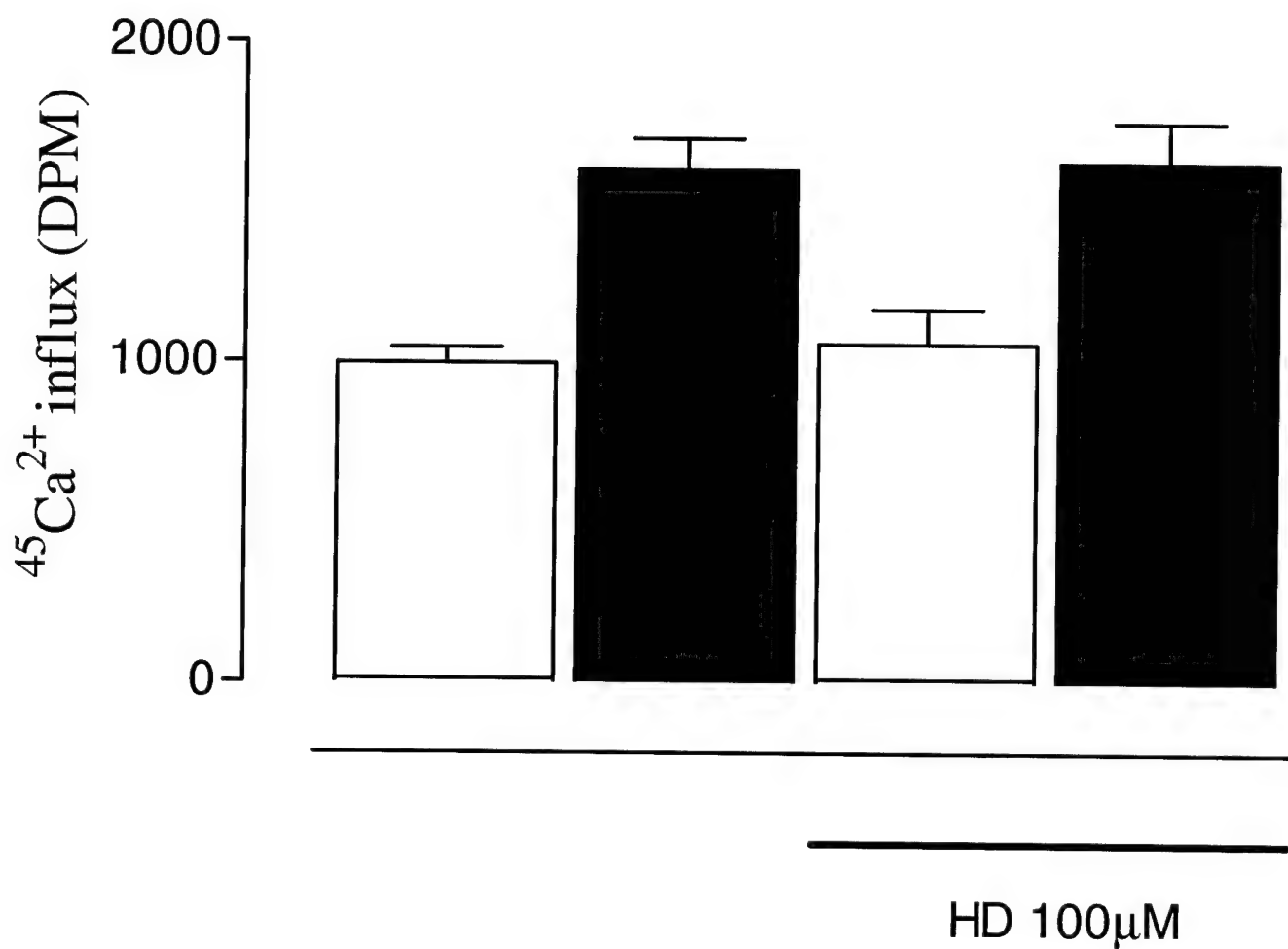
Figure 17

Lack of Effect of HD on ATP-Evoked $^{45}Ca^{2+}$ Influx

The presence of HD at 100 μM had no effect
on the ATP stimulated $^{45}Ca^{2+}$ influx (means ± SEM; n=3)
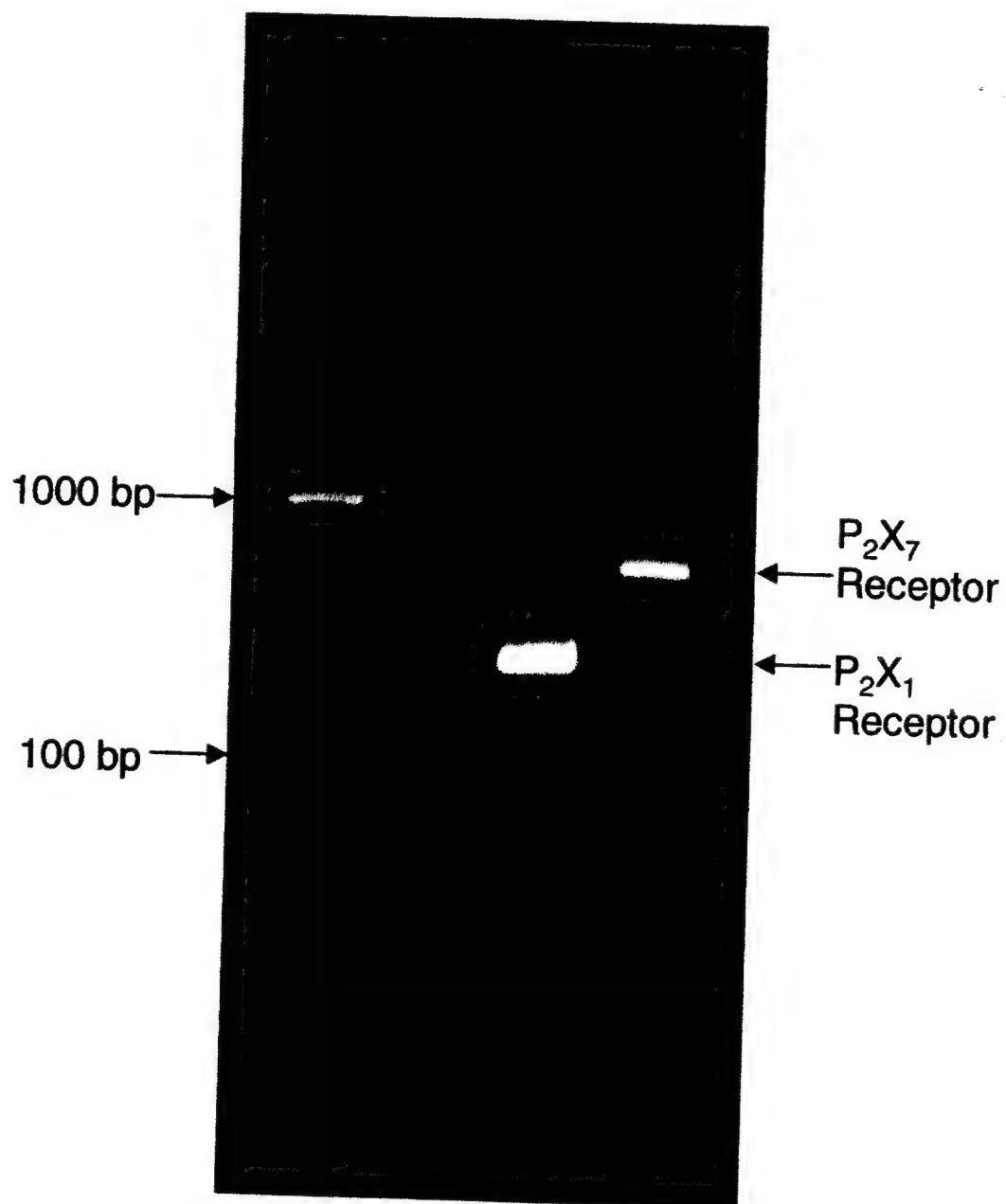
Figure 18

Expression of mRNA for P_2X_1 and P_2X_7 in Rat Vas Deferens

Total RNA was extracted from rat vas deferens and RT-PCR performed as in Methods. Photograph is of the ethidium bromide stained 693 bp and 434 bp products specific for P_2X_7 and P_2X_1 respectively.
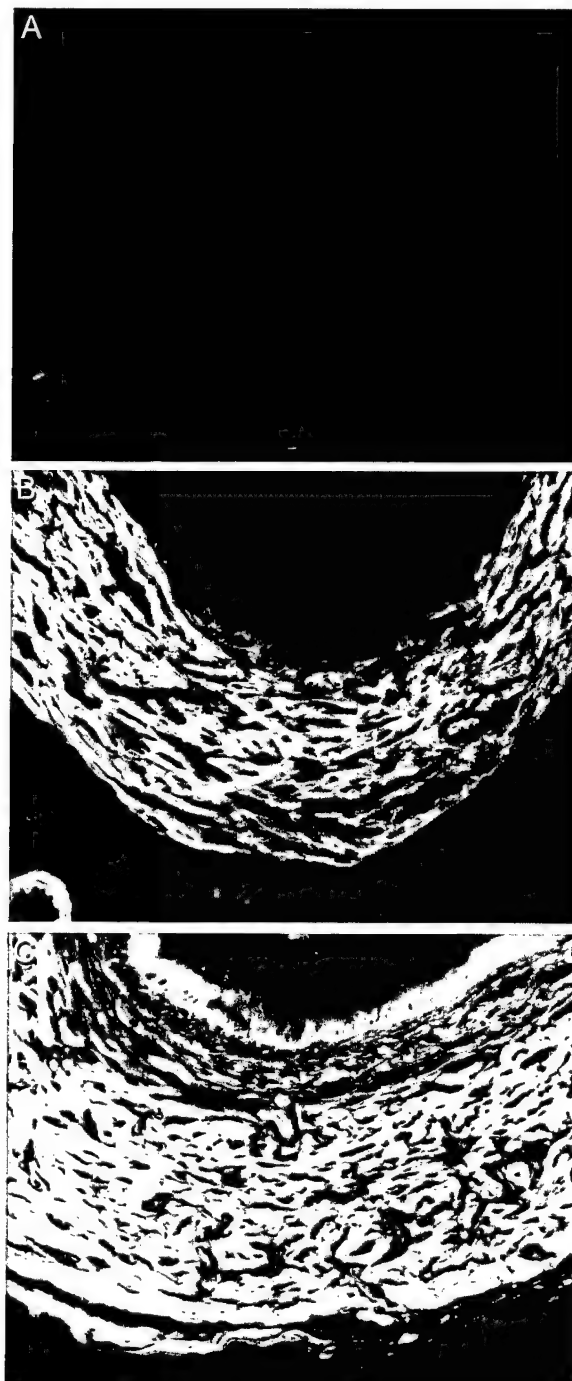
Figure 19

Immunohistochemical Detection of P2X Receptor Expression in Rat Vas Deferens

Thin sections of whole rat vas deferens were incubated with anti-P2X antibodies. Tissues stained positively (green fluorescence) for both P_2X_1 (B) and P_2X_7 (C) purinoreceptors. Control slides were negative (A).
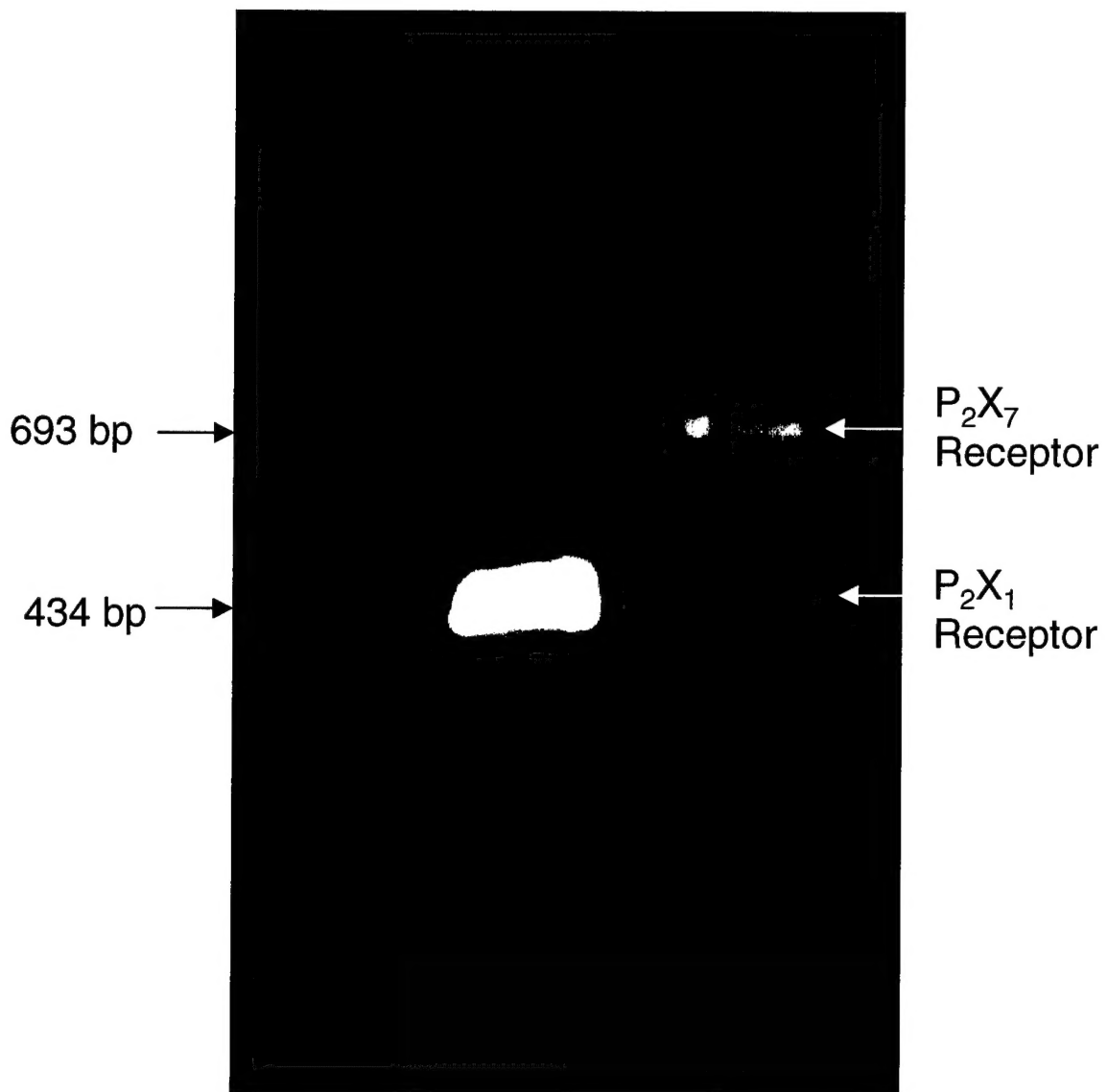
693 bp ⟶   ⟵ P_2X_7 Receptor

434 bp ⟶   ⟵ P_2X_1 Receptor

Figure 20

Expression of mRNA for P_2X_1 and P_2X_7 in Rat Thymocytes

Total RNA was extracted from rat thymocytes and RT-PCR performed according
to the procedure under Material and Methods. The photograph represents
the ethidium bromide stained 434 bp and 693 bp PCR products
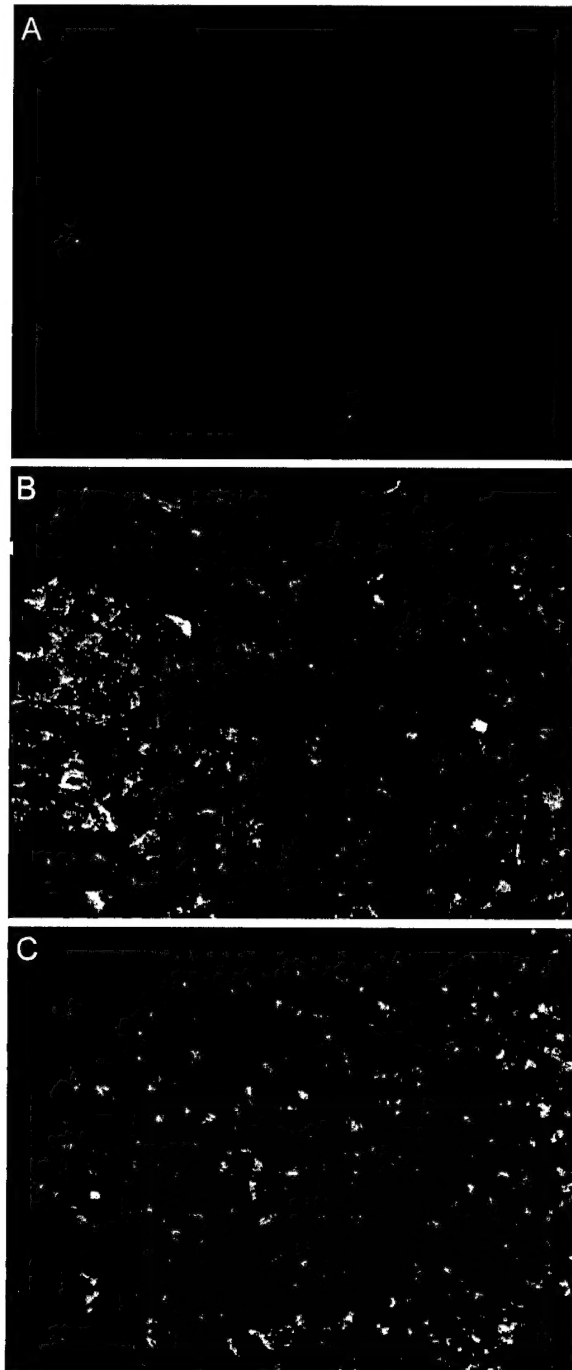specific for P_2X_7 and P_2X_1 respectively

Figure 21

Immunohistochemical Detection of P2X Receptor Expression in Rat Thymus

Thin sections of whole rat thymus were incubated with anti-P2X antibodies. Tissues stained positively (green fluorescence) for both P_2X_1 (B) and P_2X_7 (C) purinoreceptors. Control slides were negative (A).
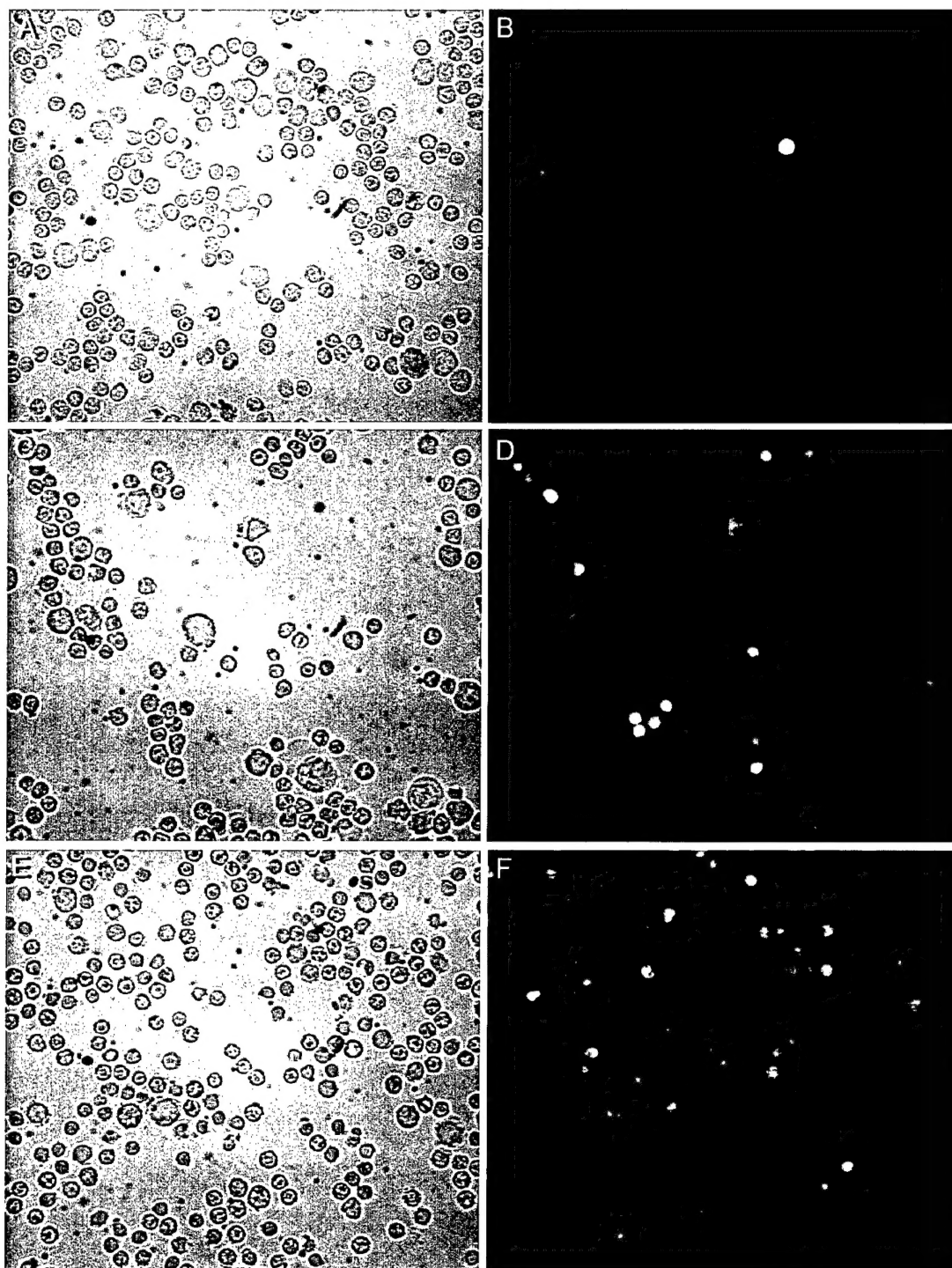
Figure 22

ATP-Mediated Uptake of YOPRO-1 by Rat Thymocytes

Isolated rat thymocytes were incubated with YOPRO-1 in the presence of 0 mM (A,B), 1 mM (C,D), and 10 mM (E,F) ATP. Fluorescent dye uptake increased with ATP concentration. Images A, C, and E: brightfield. Images B, D, and F: fluorescence.
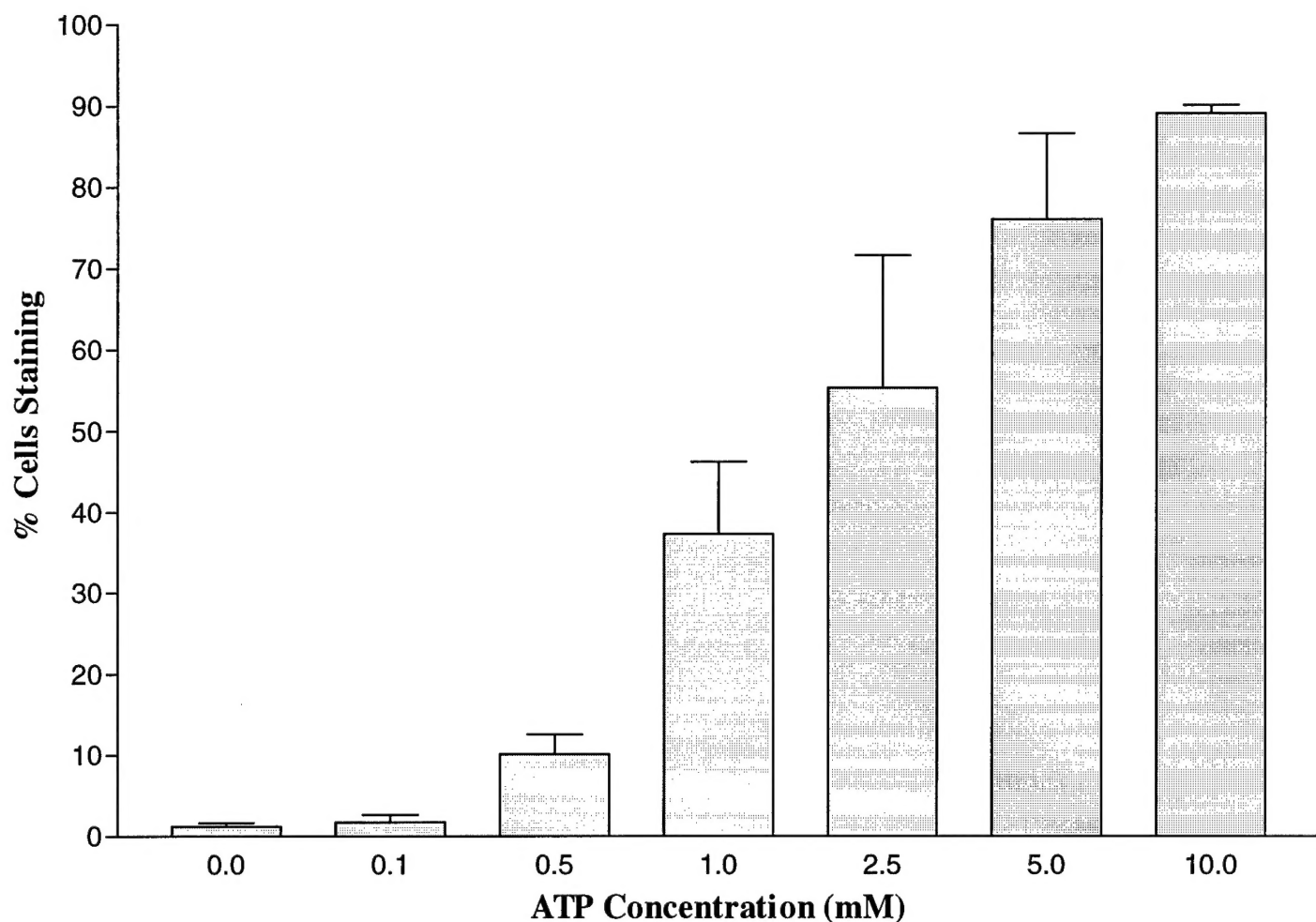
Figure 23

ATP-Mediated Uptake of YOPRO-1 by Rat Thymocytes

These results illustrate the increased staining of the thymocytes by YOPRO following a 10 min incubation with ATP. The percentage of thymocytes staining was determined by actual cell count (stained / brightfield). The data represent the mean ± SEM of 3 separarate experiments in triplicate.